Decolourisation of Selected Dyes by Lettuce and Mung Bean Seedlings: A Potential Phytoremediation Strategy

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology in the School of Biological Sciences Department

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2019

Abstract

Dye pollution is a serious environmental hazard of increasing concern due to industrialisation and increased use of synthetic dyes, especially in the textile industry. Many of these dyes are highly toxic, exhibiting carcinogenic and mutagenic effects. They also persist in the environment for long periods of time, and most current wastewater treatments are ineffective due to the recalcitrant nature of these dyes. There is a lack of research on the utilization of plants for dye removal – a process named phytoremediation.

In this thesis, I investigated the potential of fast germinating plants, including lettuce (*Lactuca sativa* L.) and mungbean (*Vigna radiata*), for the phytoremediation of environmental pollutants such as toxic synthetic dyes; malachite green (MG) and methylene blue (MB).

This study found that 10 24-hour old seedlings of *L. sativa* were capable of 96% decolourisation of MB at 0.04 mg/mL, and 87% decolourisation of MG at 0.1 mg/mL over 6 days. Three 24-hour old seedlings of *V. radiata* were capable of 85% decolourisation of MB at 0.04 mg/mL and 96% and 99.5% decolourisation of MG at 0.1 mg/mL and 1 mg/mL respectively.
Spent water from *L. sativa* or *V. radiata* seed germination collected over 8 days was also shown to be effective for decolourisation of both dyes. Spent water from *V. radiata* showed 81% decolourisation of MG (1 mg/mL) with no difference under sterile or non-sterile conditions, and 60% decolourisation of MB (0.04 mg/mL). Spent water from *L. sativa* seeds was shown to decolourise MB (0.04 mg/mL) by 72%.

Increased peroxidase activity and lipid peroxidation in *V. radiata* seedlings exposed to MB and MG were observed along with decreased radicle growth after 3 days exposure. Spectral scans of decolourised dyes compared to original solutions confirmed the absence of MB and MG and suggest degradation of the dyes has occurred. Phytotoxicity assays using lettuce seeds showed lessened toxicity of decolourised solutions.

Overall, *L. sativa* and *V. radiata* have been shown to be effective for the decolourisation of synthetic dyes, malachite green and methylene blue. It seems that they have potential for use in phytoremediation of these dyes. Both dye adsorption and biodegradation are possible mechanisms implicated in the decolourisation of the two dyes by mungbean and lettuce seedlings.

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Acknowledgements

This work would not be possible without the expert guidance and continuous encouragement and support from my senior supervisor, Associate Professor David Leung. Thank you for making the research process enjoyable and stimulating my interest in plant biotechnology. Your positivity and humour has definitely helped alleviate the stress of the past year. Thank you also to my secondary supervisor, Dr Ricardo Bello Mendoza, who gave me some good ideas to consider for my discussion.

I sincerely thank Sabai Saw Shwe and Trang Nguyen, who were extremely helpful with my data analysis for this research. My appreciation also goes out to the rest of our lab group for their constant friendliness and helpful manner inside and outside the lab this year. I also thank Sarah Kessans and Michael Love for their help with the spectroscopy analysis, and Craig Galilee, our lab floor manager, who is always willing to help solve any technical issues and has assisted me on many occasions.

I would like to take this opportunity to thank my friends and family, who have been incredibly caring and supportive this past year, especially in the last few months. Their love and encouragement has been invaluable and I feel extremely lucky to have such amazing people in my life.

The School of Biological Sciences at the University of Canterbury has a very warm and encouraging environment, it has been a pleasure to be a part of it throughout my postgraduate study.

Abbreviations

dH_2O: Distilled Water
H_2O_2: Hydrogen Peroxide
KPO_4: Potassium Phosphate
MB: Methylene Blue
MDA: Malondialdehyde
MG: Malachite Green
TBA: Thiobarbituric Acid
TBARS Assay: Thiobarbituric Acid Reactive Substance Assay
TCA: Trichloroacetic Acid

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1. Introduction and Literature Review

1.1 Dye Pollution – A Rising Global Problem

Increased production and use of synthetic dyes due to industrialisation is greatly contributing to the decline in water quality worldwide. Textile, paper, pharmaceutical, and cosmetics industries all contribute to water pollution through their use of dyes, of which large volumes are subsequently lost to the environment through effluent (Pereira & Alves, 2012). The textile industry in particular is one of the worst contaminators. Up to 10-50% of colorants (dyes) are lost to the environment through effluent during the textile dyeing process (Przystaś et al, 2012).

Global production of synthetic dyes has increased to over 7×10^5 tons per year (Drumond Chequer et al, 2013). Synthetic dyes are in high demand due to their low cost and ease of manufacture (Bafana et al, 2011) They are also preferred over natural dyes for their favourable qualities, including: a significantly larger and more vivid range of colours (Anlinker, 1977), being less prone to fading, and antimicrobial activity which is useful in textiles as dyed natural fibres will last longer (Bafana et al, 2011). While these features of synthetic dyes benefit industries who employ their use, they also contribute to the recalcitrant nature of dyestuffs lost to the environment, including their resistance to standard wastewater treatments (Rauf & Ashraf, 2012).

Dye-containing effluent contaminates water with colour, blocking sunlight from reaching aquatic species which dramatically impacts ecosystems by limiting the level of photosynthesis production, and reducing oxygen solubility through an increasing chemical oxygen demand (COD) (Vikrant et al, 2018; Yassen & Scholz, 2018). Toxicity of dyes can induce acute or chronic effects upon aquatic and terrestrial species (Pereira & Alves, 2012). For these reasons, it becomes increasingly more important to remove these harmful pollutants from the environment.

1.2 Current Dye Removal Strategies and Recent Progress

Over the years there has been significant research into various strategies for dye remediation, such as: adsorption, ion-exchange, and photochemical methods (Marimuthu et al, 2013). However, the majority of these approaches are very expensive or inefficient and many are environmentally counterintuitive due to their chemical nature, and formation of sludge or other by-products (Gürses et al, 2016; Khandare & Govindar, 2015).

1.2.1 Bioremediation

Recently, bioremediation, the use of microorganisms to degrade toxic substances, has become a popular approach for research regarding dye pollution. Many studies have shown the potential of various bacteria (Wang et al, 2011; Meerbergen et al, 2017; Karim et al, 2018), microalgae (Daneshvar et al, 2007), and fungus (Baraparte et al, 2017; He et al, 2018; Krishnamoorthy et al, 2018) to remediate various dyes or textile effluent. While this approach has many benefits, including low cost, being eco-friendly, and no sludge generation or use of chemicals (Solis et al, 2012; Kuppusamy et al, 2015; Vikrant et al, 2018), bioremediation remains limited due to the antimicrobial nature of many synthetic dyestuffs, practical complications regarding sensitivity to abiotic factors such as pH and nutrient concentrations on site (Khandare & Govindwar, 2015), and the risk of bacteria potentially metabolising dyes into possibly even more toxic metabolites (such as aromatic amines) (Platzek et al, 1999).

1.3 Phytoremediation

An emerging strategy with several benefits over bioremediation, is phytoremediation – the utilisation of plants to remove and/or degrade pollutants from soil or water (Tahir et al, 2015). The process works by plants absorbing contaminants through their roots (phytoextraction) and transporting them through the vascular system to plant tissues where they are either stored (phytostabilization), or degraded into harmless metabolites (phytotransformation) by various enzymes (Tahir et al, 2015). Certain plants, named hyperaccumulators, are preferred for phytoremediation, as their fast growth allows them to bio-accumulate pollutants more efficiently than slow growing plants (Tahir et al, 2015).

Phytoremediation for dye removal is extremely attractive due to the low cost, low-maintenance, and environmentally friendly nature of a carbon-neutral, solar-driven strategy as well as being aesthetically pleasing (Rauf & Ashraf, 2012). However, there are limited studies on the potential decolourisation and degradation of synthetic dyes by plants. *Blumea malcolmii* has been reported to decolourise 5 different dyes, including malachite green (Kagalkar et al, 2009). The phytoremediation potential of aquatic plant *Hydrocotyle vulgaris* (marsh pennywort) has been demonstrated against basic red 46 (Vafaei et al, 2013). *Aster amellus* Linn and *Alternanthera philoxeroides* (alligator weed) are capable of degrading Remazol red (Khandare et al, 2011; Rane et al, 2015) and *Eichhornia* crassipes (water hyacinth) has been reported to decolourise methylene blue up to 98.42% (Tan et al, 2016).

The use of transgenic plants for phytoremediation has also been investigated. A transgenic *Arabidopsis* plant overexpressing the enzyme triphenylmethane reductase (TMR) from *Citrobacter* sp. has displayed enhanced tolerance towards two triphenylmethane dyes, crystal violet and malachite green (Fu et

al, 2012). Another study used hairy root induction of *Sesuvium portulacastrum* (sea purslane) to enhance remediation of reactive green 19A-HE4BD (Lokhande et al, 2015). Transgenic approaches can enhance natural phytoremediation abilities of plants, but are not always a viable option as genetically modified organisms (GMO's) are strictly regulated in many countries, including New Zealand (Hayward, 2005). Therefore, transgenic plants would not be allowed into the environment for on-site phytoremediation of polluted sites.

The main disadvantages of phytoremediation are the length of treatment compared to conventional methods, vulnerability of plants to high concentrations of dyes or toxic substances, and limitations due to plant mass and root length (Tahir et al, 2015). However, the extremely low cost, and green nature of phytoremediation makes it a promising and feasible approach for the clean-up of dye pollution.

1.4 Toxic Effects of Dyes

Azo dyes are the most widely used synthetic colorant, contributing 70% to the global dye consumption of 9.9 million tons (Rawat et al, 2018). Many have been shown to be carcinogenic and mutagenic (Pereira & Alves, 2012). For this reason, there are high numbers of banned azo dyes. However, many are still used especially in underdeveloped countries due to their significant benefits including low cost (Bafana et al, 2011). Even azo dyes that are thought to be non-toxic can exhibit toxic effects due to their susceptibility to form aromatic amines through the reduction of their azo bond (Pinheiro et al, 2004). Aromatic amines have been of concern regarding dye pollution due to their acute toxicity, since the mid-1900's (Anlinker, 1977). Bioactivation of azo dyes (metabolization of aromatic amines) in humans can occur through intestinal (Flandroy et al, 2018) and skin (Platzek et al, 1999) microbiota.

1.4.1 Malachite Green

Malachite green (MG) is a triphenylmethane azo dye known to be carcinogenic and mutagenic (Banat et al, 1996). MG, the first manmade azo dye characterised by its bright green hue, is banned in several countries due to its toxicity. However, it is still widely used as a biocide in aquaculture, and is also used in the textile industry (Srivasta et al, 2004). MG can easily be absorbed in fish tissues where they are metabolised to leuco-forms (leucomalachite green). These have been shown to persist in fish tissues for several months and can be metabolised back to the original dye form which is more toxic. MG has also been shown to decrease cell viability in several human cell lines (Stammati et al, 2005). It is a multi-organ toxin and highly cytotoxic to mammalian cells (Kovacic & Somanathan, 2014). Studies have found that concentrations of just 0.1-10mg/mL MG present a significant threat towards human health (Gavrilenko et al, 2019).

1.4.2 Methylene Blue

Methylene blue (MB) is a basic dye with various applications, especially in textiles (Upendar et al, 2017). It is also used in medicine as a potential treatment for neurodegenerative disorders, and has recently been of interest as a potential antimalarial agent (Kovacic & Somanathan, 2014). Although it is not regarded as extremely hazardous, it has the ability to cause several detrimental effects in humans including vomiting, cyanosis, jaundice, quadriplegia, and tissue necrosis among others (Gupta et al, 2016; Yi & Zhang, 2008).

Both methylene blue and malachite green are cationic dyes which exhibit high toxicity to mammalian cells and severe irritation on contact to eyes and skin (Kushwaha et al, 2014; Torok et al, 2015). The harmful effects of these dyes highlight the importance in removing them from the environment.

Several studies have used plant material for the adsorption or desorption for MB or MG removal, including potato (*Solanum tuberosum*) plant waste (Gupta et al, 2017), and a powdered form of brown macroalga (*Nizamuddinia zanardinii*) (Daneshvar et al, 2017). These approaches are more environmentally friendly, and less expensive than other physio-chemical adsorption methods, such as the use of hydrogels (Hu et al, 2018) or metal-organic frameworks (MOF) (Luo et al, 2017). However, while adsorption is a successful strategy to remove toxic substances from the environment but the lack of degradation means the substance retains its highly toxic state and could potentially end up back in the environment.

The use of bacteria and other microorganisms for remediation can potentially result in metabolism of harmful metabolites if not fully degraded and risks of introducing foreign bacterial species into environments. Phytoremediation is a promising approach for dye removal, as it does not have these limitations, and remains a low-cost and eco-friendly approach (Tahir et al, 2015).

There are many other studies on adsorption (Yi & Zhang, 2008; Kushwaha et al, 2014; Agarwal et al, 2016) and bioremediation (Daneshvar et al, 2007; Wang et al, 2011; Baraparte et al, 2017; Shanmugan et al, 2017; Upendar et al, 2017; Bharti et al, 2019; Shang et al, 2019) of both malachite green and methylene blue, there is a significant lack of research on phytoremediation for these dye species, despite the many advantages of this approach.

1.5 Oxidative Stress

While even small concentrations of dye can be toxic, plants demonstrate resistance through various mechanisms. Peroxidases are enzymes involved in many cellular processes, including the removal of damaging reactive oxygen species (ROS) that are formed during oxidative stress and are also known to be involved in the degradation of synthetic dyes (Kalsoom et al, 2015).

Several studies have performed enzyme assays to measure antioxidant levels in plants exposed to synthetic dyes and have all reported a significant increase in peroxidase activity in a concentration dependent manner (Vafaei et al, 2013; Watharkar & Jadhav, 2014; Rane et al, 2015). These studies all suggested that this increase in enzyme activity may be linked to the mechanism in which decolourisation and degradation of the dyes occur.

1.6 Lettuce and Mung Bean

Lettuce (*Lactuca sativa* L.) and mungbean (*Vigna radiata*) are both common plants, with fast germination and growth, making them ideal candidates for this research. Both plants have been found to contain many antioxidants, and other health-promoting compounds (Kim et al, 2016; Ganesan & Xu, 2018). There are some previous studies demonstrating the effective use of L. *sativa* phytoremediation for the removal of phenol, a highly toxic compound, (Tadic et al, 2018), and Chromium (Cr) VI, the most hazardous form of Cr (Dias et al, 2016). The ability of this lettuce species to survive when exposed to such highly toxic compounds, and maintain its phytoremediation activity, is promising for its use in the current study. Mungbean is a relatively untested plant species for phytoremediation, however, due to its fast growth and high antioxidant activity (especially during the sprouting process) it is also a promising plant for the use of dye decolourisation in this study.

1.7 Aims and Objectives

There is a shortage of information regarding phytoremediation of malachite green and methylene blue; two well-known toxic synthetic dyes. With the short time available for MSc. experimental work in the lab, it was hypothesised that the use of fast germinating seeds could be useful in aiding investigations into some aspects of phytoremediation of these dyes.

In this research, the aim was to determine if fast germinating plants, including mungbean and lettuce, would be useful in phytoremediation of environmental pollutants such as toxic synthetic dyes; malachite green and methylene blue.

Specific objectives of this research were:

1. Determination of several basic parameters in relation to the decolourisation, by lettuce and mungbean seedlings, of malachite green and methylene blue dissolved in distilled water in the lab;

2. Determination of the relative effectiveness of lettuce compared to mungbean seedlings in the decolourisation of malachite green and methylene solutions;

3. Decolourisation of dyes dissolved in water that were used to imbibe lettuce and mungbean seeds;

4. Comparison of decolourisation of dyes under sterile and non-sterile conditions;

5. Determination of antioxidant activity in lettuce and mungbean seedlings in response to oxidative stress resulting from dye exposure; and

6. Seeking some evidence including phytoxicity bioassay and spectrophotometric scanning for a substantial reduction in toxic substances in the dye solutions by the lettuce and mungbean seedlings.

Phytoremediation using lettuce and mungbean may prove to be an effective method for the removal of dyes, with several benefits over alternative methods such as adsorption and bioremediation which are predominant in the current research.

2. Materials and Methods

2.1 Plant Selection

Lettuce seeds (*Lactuca sativa* L.) of the Great Lakes variety were obtained from King Seeds (New Zealand) and stored in a fridge kept at 4°C.

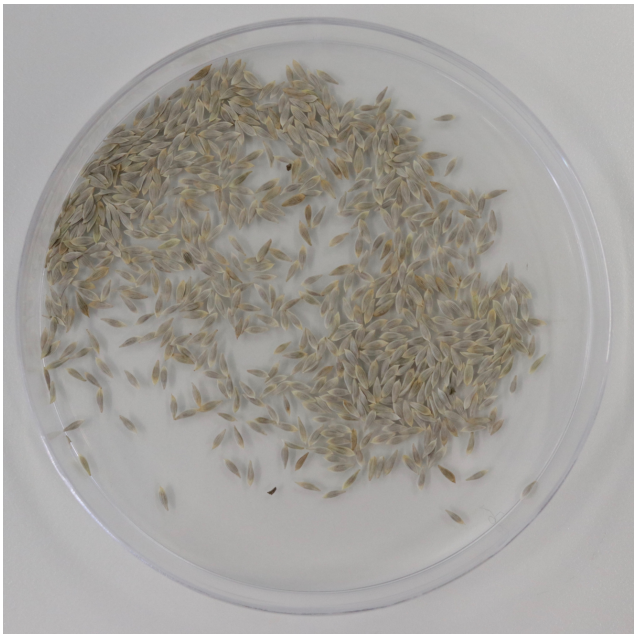

Figure 1: Lettuce (*Lactuca sativa* L.) seeds of the Great Lakes variety. [Photo taken by Denver Adams (2019)]

Mungbean seeds (*Vigna radiata*) were obtained from a local supplier and kept within sealed plastic containers in a dark cupboard at room temperature.

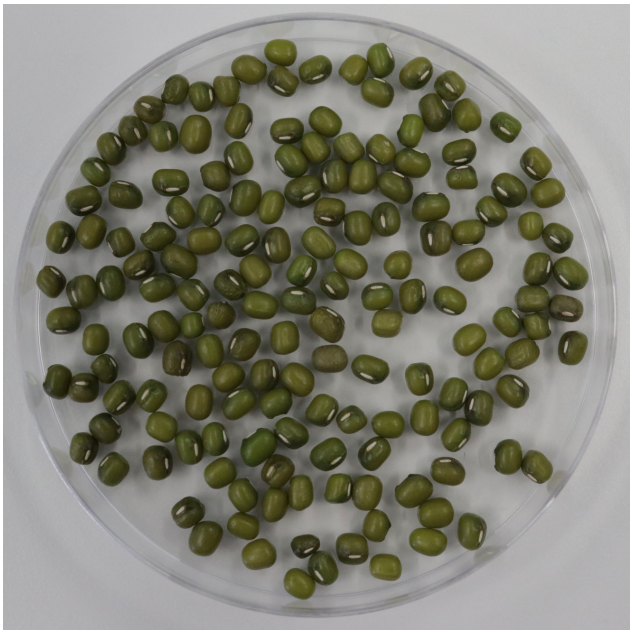

Figure 2: Mungbean seeds (*Vigna radiata).* [Photo taken by Denver Adams (2019)]

2.2 Dye Selection

2.2.1 Methylene Blue

Methylene blue dye used in this study was purchased from Sigma-Aldrich.

- IUPAC Name: [7-(dimethylamino)phenothiazin-3-ylidene]-dimethylazanium;chloride
- Molecular Formula: $C_{16}H_{18}ClN_3S$
- Molecular Weight: 319.851 g/mol
- Maximum Absorbance: 670nm

A stock solution of 0.4 mg/mL was prepared and stored in a dark cupboard at room temperature.

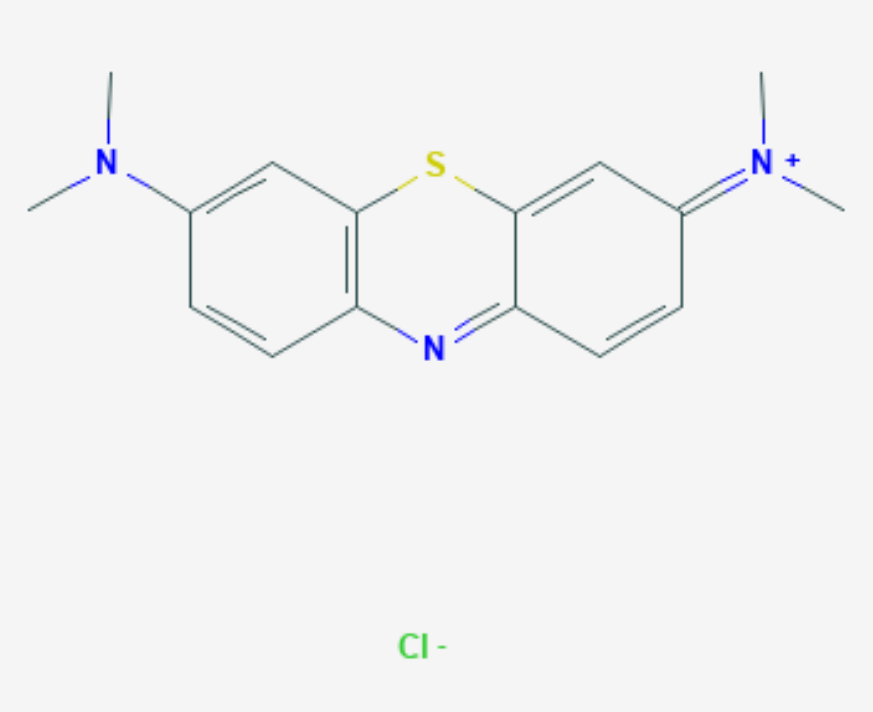

Figure 3: 2D Structure of methylene blue obtained from PubChem [https://pubchem.ncbi.nlm.nih.gov]

2.2.2 Malachite Green

Malachite green dye used in this study was purchased from Sigma-Aldrich.

- IUPAC Name: [4-[[4-(dimethylamino)phenyl]-phenylmethylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium;chloride
- Molecular Formula: $C_{23}H_{25}ClN_2$
- Molecular Weight: 364.917 g/mol
- Maximum Absorbance: 619nm

A stock solution of 10 mg/mL was prepared and stored in a dark cupboard at room temperature.

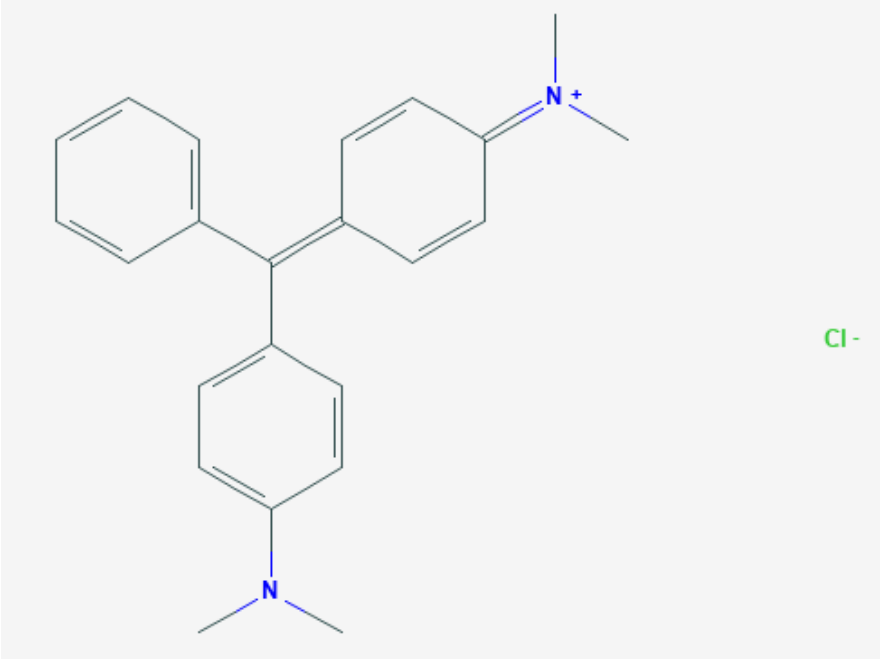

Figure 4: 2D structure of malachite green obtained from PubChem [https://pubchem.ncbi.nlm.nih.gov]

2.3 Preliminary Experiment

2.3.1 Toxicity Screening of Dyes on Seeds

Ten lettuce seeds and five mungbean seeds (per tube) were placed in glass universal tubes containing 5 mL of each respective dye at a particular concentration (0.01, 0.1 and 10 mg/mL of malachite green; 0.04, 0.1 and 0.4 mg/mL of methylene blue) along with dH_2O as a control.

Germination percentages for each treatment were determined after 8 days of sowing seeds in dH_2O or a dye solution at room temperature.

2.4 Decolourisation Experiments

2.4.1 General Procedure

All decolourisation experiments were performed in triplicates within glass universal tubes each containing 5 mL of respective treatments (dye or dH_2O). Seeds of both lettuce and mungbean were left to germinate over 24 hours at room temperature before being placed in solutions and then left in an incubator at 23°C for a certain time duration (specified for each experiment).

Two 1 mL-aliquots were taken from each sample after decolourisation and transferred into Eppendorf tubes. These were centrifuged at 10,000 rpm for 1

minute, supernatants retained which were diluted if necessary. The absorbance of malachite green test solutions of 1 and 0.1 mg/mL after 1:50 dilution and 3:10 dilution, respectively, was measured. A SPECTRAmax M5 plate reader was used to measure absorbance at 619 nm for MG samples, and 670 nm for MB samples. Decolourisation percentage was calculated using the below formula:

Decolourisation % = [(initial absorbance – final absorbance)/initial absorbance] x 100

2.4.2 Effect of Varying Seedling Number

A range of 1-5 mungbean seedlings were placed in tubes containing 1 mg/mL malachite green or 0.04 mg/mL methylene blue. Controls used were dH_2O , and the same dyes with no plant material. All the tubes were placed in an incubator with continuous lighting but some of the tubes were wrapped in tinfoil with no light penetration as treatments kept in the dark.
After 6 days of incubation, decolourisation percentages were determined.

2.4.3 Effect of Varying Incubation Time

Three mung bean seedlings were placed in tubes containing malachite green (1 mg/mL). MG (1 mg/mL) and dH_2O with no plant material were used as controls. All treatments were kept under both light and dark conditions.

Incubation duration was varied from 3 to 9 days. At the end of each incubation duration, decolourisation percentages were determined.

2.4.4 Dye Decolourisation Using Lettuce and Mung Bean: A Comparison

Both lettuce (10 seedlings) and mungbean (3 seedlings) were placed in 1 mg/mL and 0.1 mg/mL malachite green, and 0.04 mg/mL methylene blue for 6 days under light conditions. Dyes at each concentration, and dH_2O with no plant material were used as controls. After 6 days, decolourisation for all test solutions was recorded.

2.5 Spent Water Decolourisation

2.5.1 General Procedure

In these experiments dH_2O was used to incubate lettuce or mungbean seeds for some time and then the germinated seeds were removed. The water obtained or called 'spent water' here, was used to prepare both malachite green (1 mg/mL) and methylene blue (0.04 mg/mL). The spent water was used in the dilution of concentrated dyes (10 mg/mL MG and 0.04 mg/mL MB) to reach the desired working concentration. Fresh dye of each type was prepared from dH_2O to serve as the control for these experiments.

In all other aspects, the same procedure for the above decolourisation experiments was followed (refer to **2.4.1**).

2.5.2 Effect of Varying Seed Number

Lettuce (10 and 20) and mungbean (3 and 6) seeds were placed in 5 mL dH_2O in an incubator at 23°C for 4 days. Plant material was removed and the spent water from each respective treatment was used to dilute concentrated malachite green to 1 mg/mL and methylene blue to 0.1 mg/mL. These samples, along with dH_2O prepared dye controls, were placed back into the incubator for a further 4 days before decolourisation percentages were measured.

2.5.3 Comparison of Dye Decolourisation Under Sterile and Non-Sterile Conditions

For these experiments the decolourisation percentages of two spent water treatments were measured under sterile and non-sterile conditions.

Treatment A: Spent water from 6 mungbean seedlings placed in 5 mL dH_2O for 4 days and then replaced with fresh seeds and left a further 4 days (8 days total).
Treatment B: Spent water obtained from 6 mungbean seeds placed in 5 mL dH_2O for 8 days.

Sterile Conditions: This experiment was prepared in a laminar flow hood. Seeds were surface sterilised in 30% bleach for 10 minutes, and then rinsed 4 times in sterile deionised water. Glass universal tubes and pipette tips were pre-sterilised in an autoclave (121°C at 15 psi for 20 min).

A concentrated malachite green dye solution (10 mg/mL) was filter-sterilised through a disposable pre-sterilised, 0.22 µm membrane filter (Millipore). This filter-sterilized dye was diluted by spent water from each treatment (or sterile deionised water for controls) to reach 1 mg/mL for each sample. All tubes were sealed with lids and then taken to the 23°C incubator and left for 4 days before decolourisation percentage was measured.

2.5.4 Effect of Concentrated Spent Water

Spent water was obtained from 6 mungbean seedlings, or 20 lettuce seedlings placed in 5 mL dH_2O for 4 days and then replaced with the same number of fresh seeds and left a further 4 days (8 days total).
Malachite green (0.1 mg/mL) and methylene blue (0.04 mg/mL) dyes were prepared from each spent water treatment, with dH_2O prepared dyes as controls. Decolourisation percentages were measured after 4 days of incubation at 23°C.

2.6 Oxidative Stress Experiments

2.6.1 Peroxidase Assay

Twenty-four hour old germinated lettuce (20 per tube) and mungbean (5 per tube) seedlings were placed in glass universal tubes containing 5 mL distilled H_2O, malachite green (1 mg/mL) and methylene blue (0.04 mg/mL) for both 1 and 3 days. Seedlings were removed and rinsed in dH_2O to remove any surface dye before enzyme extraction.

Peroxidase assay procedure was adapted from Baque et al. (2010). Root tips (20 lettuce, or 5 mungbean) were excised (3 mm from the root tip) and homogenised using a mortar and pestle on ice, in KPO_4 buffer (0.1M, pH 6.9) to a total volume of 1.2 mL (4 x 0.3 mL) and transferred into Eppendorf tubes. The homogenates were centrifuged at 4°C for 5 minutes at 10,000rpm. Supernatant (enzyme extract) was then transferred to a new Eppendorf tube and 50 µL of enzyme extract was added to a reaction mixture. Absorbance of the reaction mixture was measured after 5 minutes. The reaction mixture is shown below in Table 1.

Table 1: Reaction Mix for Peroxidase Assay

Solution	Treatment	Blank
KPO_4 Buffer	943 µL	993 µL
H_2O_2 (10%)	5 µL	5 µL
Guaiacol	2 µL	2 µL
Enzyme	50 µL	-

Enzyme controls were also used in this experiment. These were prepared by taking out 100 µL of the enzyme extracted from each treatment (after the centrifuge step), and boiling at 100°C for 5 minutes. Then the same volume (50 µL) of enzyme from the boiled treatment was used in the reaction mixture, instead of the unboiled enzyme extract.

Total peroxidase activity was calculated and expressed as Ab unit/min/root tip.

2.6.2 Lipid Peroxidation (TBARS) Assay

Twenty-four hour old germinated seedlings of lettuce (5 x 20 per tube) and mungbean (5 x 5 per tube) were placed in glass universal tubes containing 5 mL dH_2O , malachite green (1 mg/mL) and methylene blue (0.04 mg/mL) for both 1 and 3 days. Seedlings were removed and rinsed in dH_2O to remove any surface dye before enzyme extraction.

Lipid peroxidation-thiobarbituric acid reactive substances (TBARS) assay procedure was adapted from Hodges et al. (1999). Root tips (100 lettuce, or 25 mungbean) were excised (3 mm from the root tip) and homogenised using a mortar and pestle on ice, in 0.1% trichloroacetic acid (TCA) to a total volume

of 1.2 mL (4 x 0.3 mL) and transferred to Eppendorf tubes. Samples were centrifuged at 4°C for 5 minutes at 10,000rpm and supernatant retained.

A mixture of 0.5 mL supernatant (enzyme) and 1 mL 20% TCA containing 0.5% thiobarbituric acid (TBA), or 20% TCA only (for the blank) was prepared in a glass tube. Samples were then placed in a 85°C water bath for 25 minutes. Afterwards, the reaction solution for each sample were transferred into Eppendorf tubes and cooled on ice and then centrifuged again at 4°C for 5 minutes at 10,000rpm. Absorbance was read at 532nm.

The concentration of the malondialdehyde (MDA)-TBA complex produced was calculated using the molar extinction coefficient, ε=155 mM^{-1} and expressed as µM MDA/root tip.

2.7 Growth Measurements

Lettuce (20 seedlings) and mungbean (5 seedlings) were lined up on filter paper within a petri dish and photographed next to a reference of 1 cm before being placed in glass universal tubes containing 5mL dH_2O, malachite green (1 mg/mL) and methylene blue (0.04 mg/mL) for both 1 day, and 3 days. Each seedling was then photographed again afterwards.

Image J software was used to measure the radicle lengths of each seedling from the before and after photos, and average growth (mm) per treatment was calculated.

2.8 Phytotoxicity Evaluations

2.8.1 Direct Decolourisation

After the decolourisation of methylene blue (0.04 mg/mL) and malachite green (1 mg/mL and 0.1 mg/mL) by lettuce and mungbean (**2.4.4**), approximately 2 mL of the remaining solutions from each treatment was transferred into new tubes after the removal of all plant material. Original solutions of MB (0.04 mg/mL) and MG (1 mg/mL and 0.1 mg/mL) were used as controls, along with dH_2O. Ten lettuce seeds were placed in each tube and left for 48 hours before germination percentage and radicle growth (mm) were recorded.

2.8.2 Spent Water Decolourisation

After the decolourisation of methylene blue (0.04 mg/mL) and malachite green (1 mg/mL) by spent water from lettuce and mungbean seedlings (2.5.4), approximately 2 mL of the remaining solutions from each treatment were transferred into new tubes. Ten lettuce seeds were placed in each tube

and left for 48 hours before germination percentages and radicle growth (mm) were recorded.

2.9 Scanning Wavelength Measurements

2.9.1 Direct Decolourisation

Absorbance of malachite green (1 mg/mL and 0.1 mg/mL) and methylene blue (0.04 mg/mL) solutions and their decolourised counterparts by 10 lettuce or 6 mungbean seedlings were measured from 200 nm to 800 nm, with a 10 nm step using a SPECTRAmax M5 plate reader.

2.9.2 Spent Water Decolourisation

Absorbance of malachite green (1mg/mL) and methylene blue (0.04mg/mL) and their spent water (20 lettuce or 6 mungbean seeds replaced after 4 days for a total of 8 days) decolourised counterparts were measured from 200nm to 800nm, with a 10nm step using a SPECTRAmax M5 plate reader.

2.10 Data Analysis

Statistical data analysis for the data were carried out in both SAS and SPSS softwares. All of the data were assumed to have a normal distribution and were analyzed using one-way ANOVA, and least significant difference (LSD). Significant differences between treatments were established with $P < 0.05$.

3. Results and Discussion

3.1 Preliminary Experiments

3.1.1 Toxicity Screening of Dyes on Seeds

Increasing concentrations of methylene blue (Figure 5a) and malachite green (Figure 5b) dyes were shown to significantly decrease the germination percentage of lettuce seeds.
This screening confirmed the toxic nature of these two dyes, and was useful to establish suitable concentrations of the dyes for use in the decolourisation experiments later on.

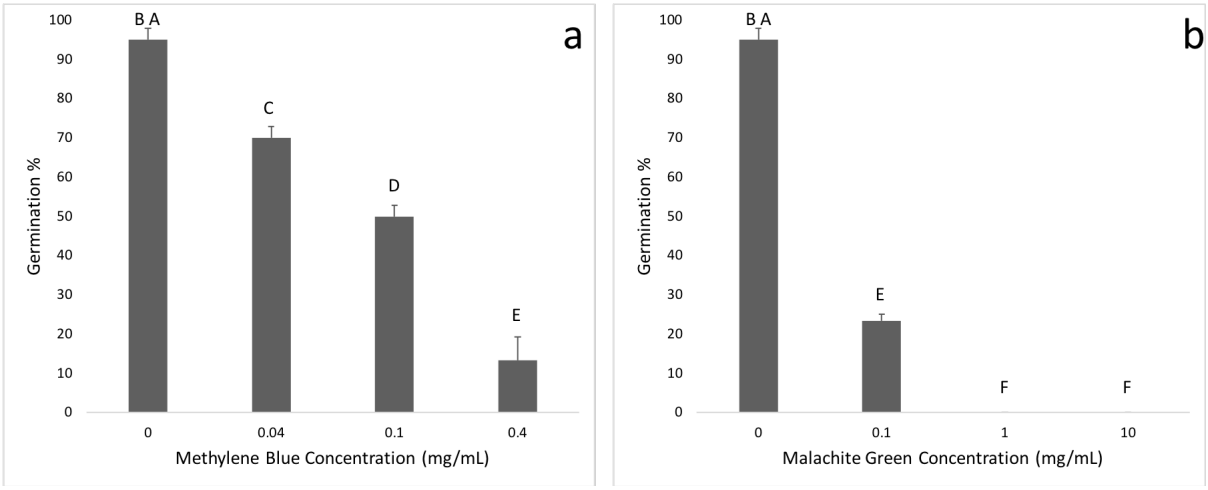

Figure 5: Effect of increasing methylene blue (a) and malachite green (b) concentrations on the germination % of lettuce seeds after 8 days. *Presented values are the mean germination percentages +SE (n=3). Means with different letters indicate that they are significantly different. LSD = 12.036, p <0.001.*

3.2 Decolourisation Experiments

3.2.1 Effect of Varying Seedling Number

Overall, the results indicate a significant increase in decolourisation of each dye in relation to seedling number, until a certain maximum decolourisation capacity is reached. The number of seedlings required to reach this peak ranges from 2-3 seedlings for all three dye solutions.

There was no significant difference between the decolourisation from light or dark conditions for malachite green (1 mg/mL and 0.1 mg/mL) solutions (Figures 7 and 8 respectively), however there was an small increase in decolourisation percentage in dark conditions for the methylene blue (0.04 mg/mL) solutions (Figure 6). Despite this difference, both light and dark conditions exhibit high decolourisation (76-85%) of MB with the most significant decolourisation occurring in treatments with 3 or more seedlings.

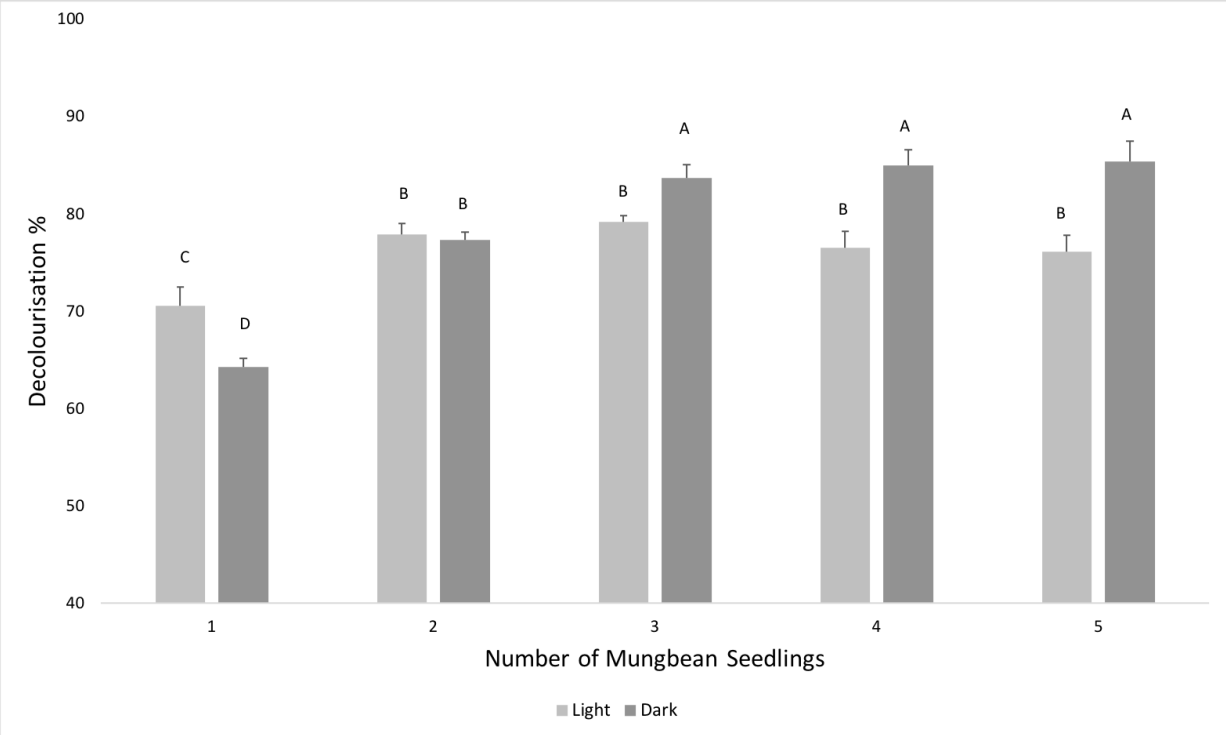

Figure 6: Effect of varying number of mungbean seedlings on the decolourisation of methylene blue (0.04 mg/mL). *Values presented are the mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 4.101, P < 0.001.*

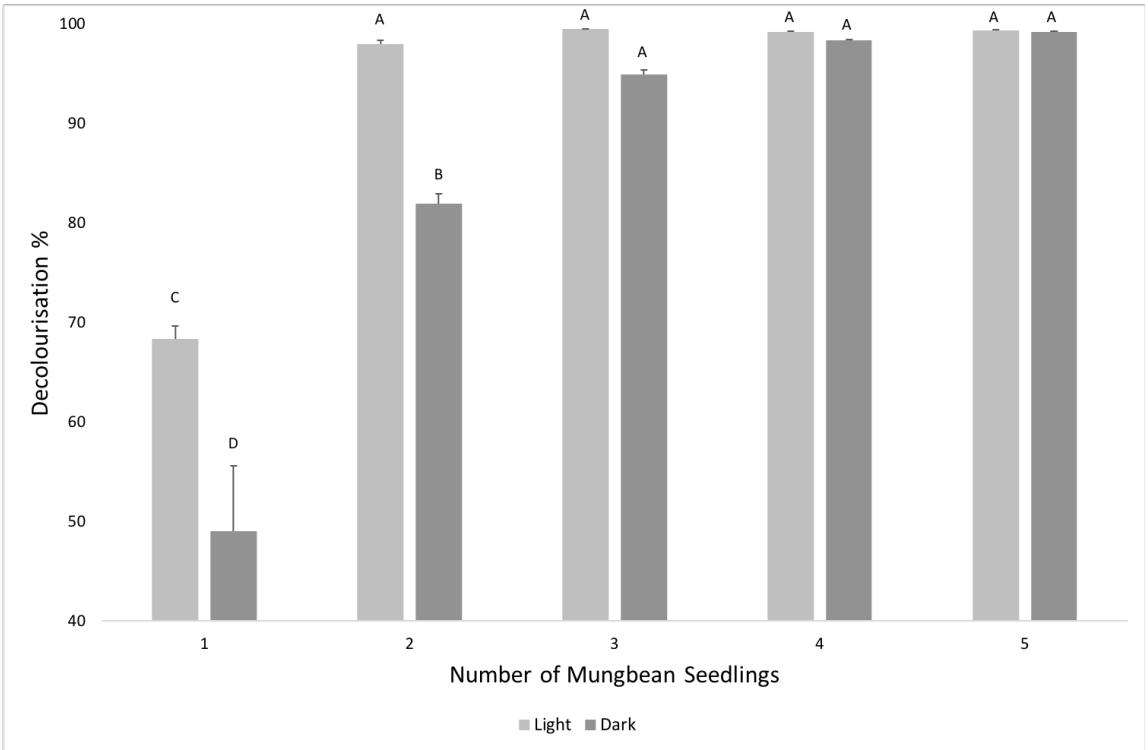

Figure 7: Effect of varying number of mungbean seedlings on the decolourisation of malachite green (1 mg/mL). *Values presented are the mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 5.975, P < 0.001.*

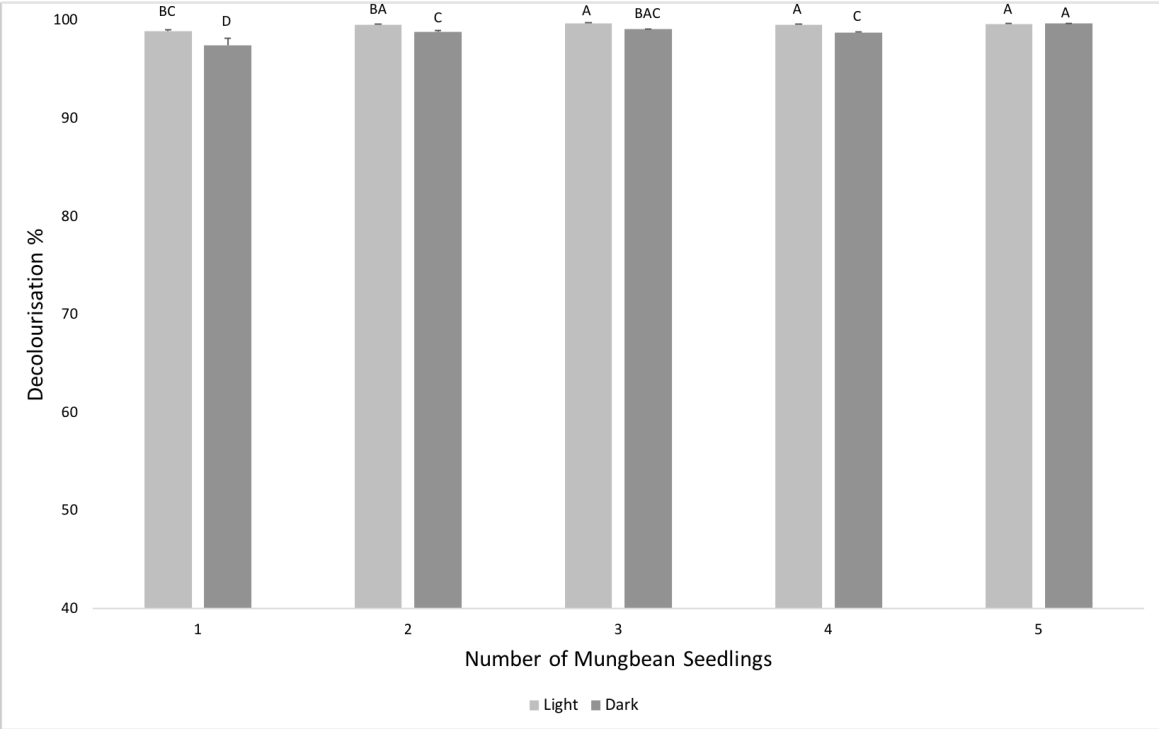

Figure 8: Effect of varying number of mungbean seedlings on the decolourisation of malachite green (0.1 mg/mL). *Values presented are the mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 0.672, P < 0.001.*

Decolourisation of MG and MB under light conditions were not significantly higher than the decolourisation observed under dark conditions (at peak decolourisation). This indicates that decolourisation of each of these dyes is not assisted by, or a result of photodegradation.

Decolourisation of malachite green at both concentrations (1 mg/mL and 0.1 mg/mL) was highest with 3 seedlings present, demonstrating over 99% decolourisation after 6 days. This high level of decolourisation from only a small number of mungbean seedlings demonstrates the effectiveness and promise of this plant species in the phytoremediation of synthetic dyes.

3.2.2 Effect of Varying Incubation Time

To investigate if the maximum decolourisation by 3 mungbean seedlings could be reached in a shorter incubation period, a time-course experiment was followed (Figure 9). The results show that maximum decolourisation (>96%) was achieved by 6 days. There is a significant increase in decolourisation percentage over 6 days compared to 3 days, but no further increase is observed with a longer incubation time. These findings suggest that the optimum mungbean seedling number and time duration for maximum decolourisation, is 3 seedlings over 6 days incubation in either light or dark conditions.

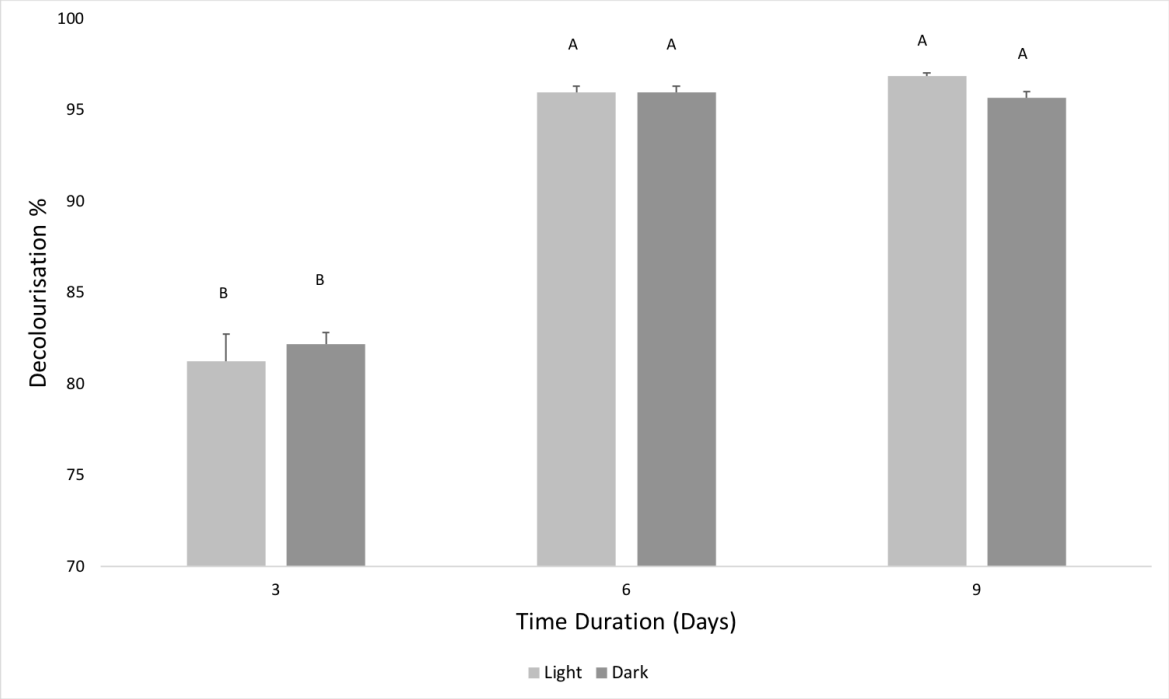

Figure 9: Effect of varying incubation time on the decolourisation of malachite green (1 mg/mL) by mungbean seedlings. *Values presented are the mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 1.994, P < 0.001.*

Both the seedling number and time duration variation experiments were important to determine some basic parameters for decolourisation of methylene blue and malachite green using mungbean seedlings. This is especially important due to lack of previous information or experimental work regarding the use of this plant species for these dyes, or any other synthetic dyes in the scientific literature, currently. Alternatively, there has been past experimental work on lettuce seedlings, which found that 10 lettuce seedlings can decolourise up to 86% MB (0.04 mg/mL) within 92 hours (Dhaneshwar, 2016). This information was applied to the current study, and either 10 lettuce seedlings, or 3 mungbean seedlings were used for each decolourisation experiment.

3.2.3 Dye Decolourisation Using Lettuce and Mung Bean: A Comparison

Decolourisation by both seedling types over 6 days were compared between both methylene blue (0.04 mg/mL) and malachite green (1 mg/mL and 0.1 mg/mL) solutions. Lettuce seedlings were shown to decolourise MB at a significantly higher capacity than mungbean seedlings, with 96% decolourisation compared to 85% by mungbean (Figure 10). This amount of decolourisation is also 10% higher than the decolourisation percentage found by Dhaneshwar (2016) over 4 days. This shows that a longer incubation time increased the decolourisation of MB (0.04 mg/mL) by lettuce seedlings.

Figure 10: Average decolourisation of methylene blue (0.04 mg/mL) after 6 days of lettuce and mungbean treatment. *Presented values are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 1.558, p < 0.001.*

Comparison of MG decolourisation by lettuce and mungbean seedlings had an inverse result, with mungbean exhibiting significantly higher decolourisation than lettuce at both concentrations. This difference was especially dramatic in malachite green (1 mg/mL) solution (Figure 11). Mungbean seedlings decolourised the solution by over 96%, a 75% increase when compared to the 19% decolourisation achieved by lettuce seedlings. However, when MG concentration was decreased to 0.1 mg/mL (Figure 12), lettuce decolourisation increased to 87% while mungbean decolourisation remained significantly higher at 99.5%.

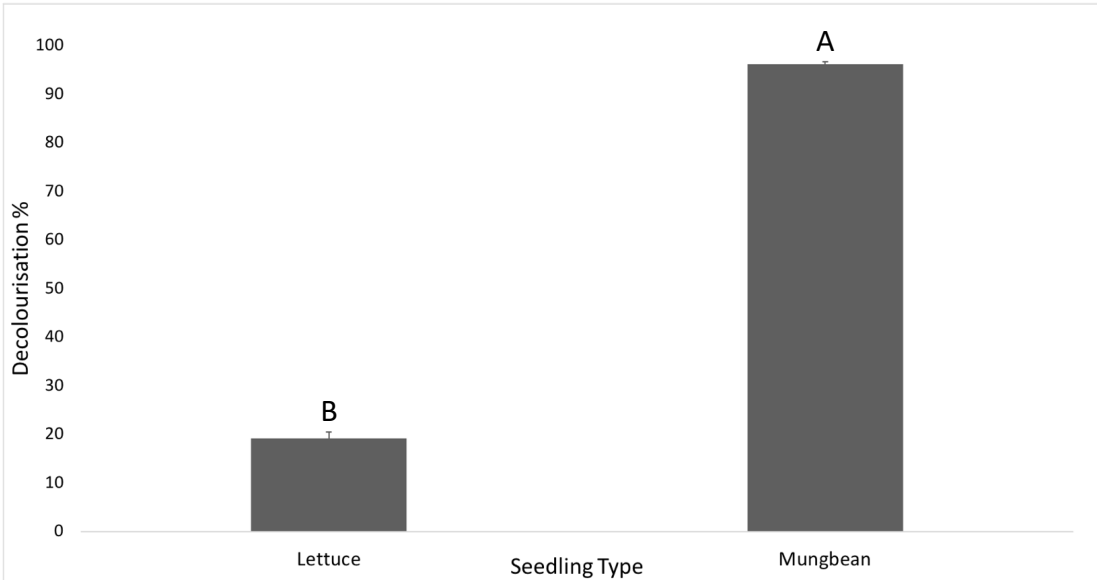

Figure 11: Average decolourisation of malachite green (1 mg/mL) after 6 days of lettuce and mungbean treatment. *Presented values are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 2.784, p < 0.001.*

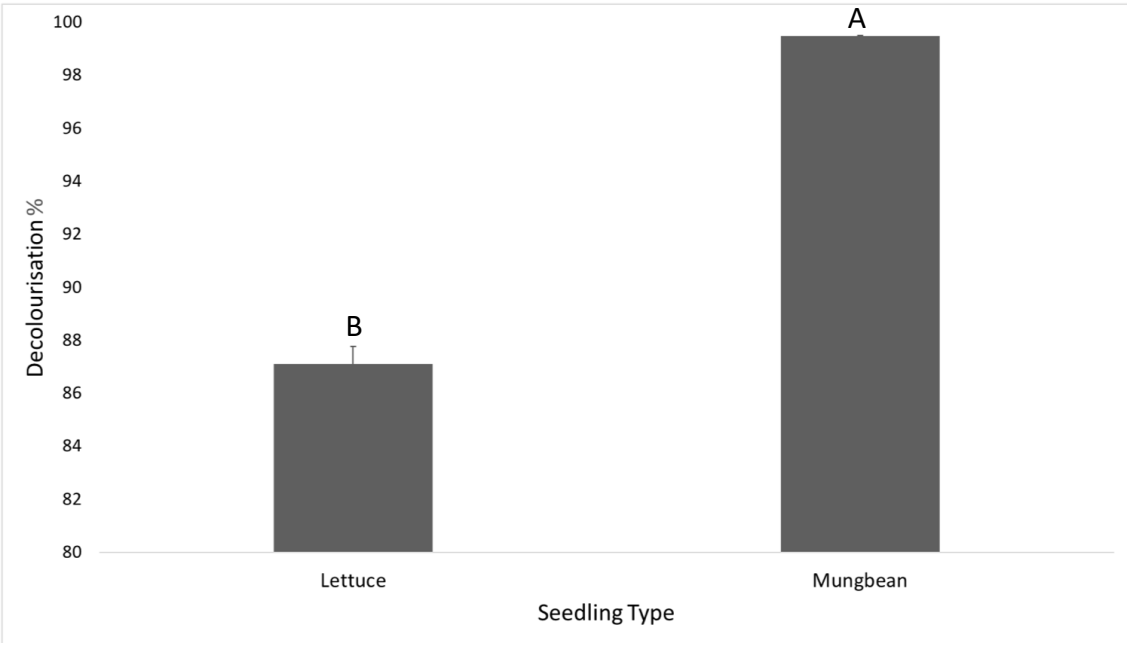

Figure 12: Average decolourisation of malachite green (0.1 mg/mL) after 6 days of lettuce and mungbean treatment. *Presented values are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 1.381, p < 0.001.*

These comparisons reveal the enhanced decolourisation capacity of lettuce seedlings for MB (0.04 mg/mL), and mungbean seedlings for MG (1 mg/mL and 0.1 mg/mL) decolourisation. Mungbean seedlings are still effective in MB decolourisation, as over 85% was reached, and lettuce seedlings were capable of 87% decolourisation in MG (0.1 mg/mL). This shows that while each seedling has an affinity for a certain dye of the two used in this study, they still demonstrate high decolourisation capability of both dyes. However, mungbean seedlings are shown to be more suitable for higher concentrations of MG. A previous study found that laccase from *Trichoderma* spp. could decolourise 0.1 mg/mL MG by 97% (Shanmugan et al, 2017). The decolourisation by mungbean seedlings of MG the same concentration, was found to be higher (99.5%) in this study. And 96% decolourisation by mungbean seedling was achieved at a dye concentration 10 times higher than the concentration used for dye removal in several other studies also (Gupta et al, 2011; Shang et al, 2019). This shows that mungbean is particularly effective for the decolourisation of higher concentrations of MG.

3.3 Spent Water Decolourisation

3.3.1 Effect of Varying Seed Number

Spent water from mungbean seedlings collected over 4 days, was able to decolourise methylene blue (0.04 mg/mL) by approximately 57.5% (Figure 13b). However, there was no apparent increase in decolourisation of this solution by increasing the number of mungbean seedlings. While there was a small increase in decolourisation between 10 and 20 lettuce seedlings (Figure

13a), it was not enough to be a significant difference, and only 12% decolourisation was observed by the spent water from 20 lettuce seedlings.

There was a significant increase in the decolourisation of malachite green (1 mg/mL) by the spent water from 6 mungbean seedlings resulting in 25% decolourisation compared to 13% from spent water of 3 seedlings (Figure 14b). Spent water from lettuce seedlings were ineffective for this dye solution, only showing 1.8% decolourisation (Figure 14a).

The findings from this experiment suggest that certain molecules/enzymes released by lettuce and mungbean into the water they were incubated in, can decolourise both dyes without either seedlings being present during decolourisation. This is consistent with the possibility that decolourisation of the dyes by the seedlings could be attributed to not only dye adsorption to the seedlings but also biodegradation.

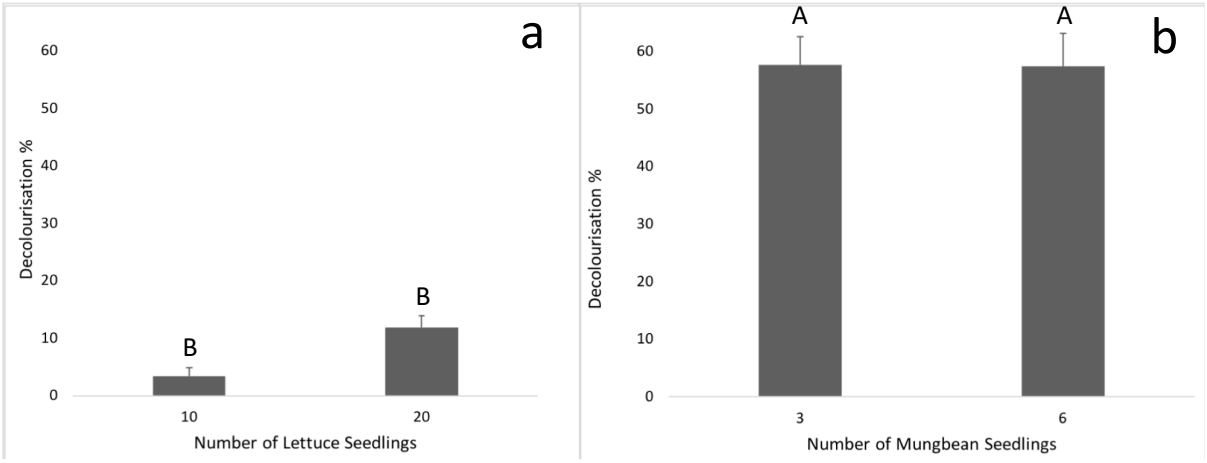

Figure 13: Effect of varying seedling number of lettuce (a) and mungbean (b) for spent water decolourisation of methylene blue (0.04 mg/mL). *Values presented are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 12.4, p <0.001.*

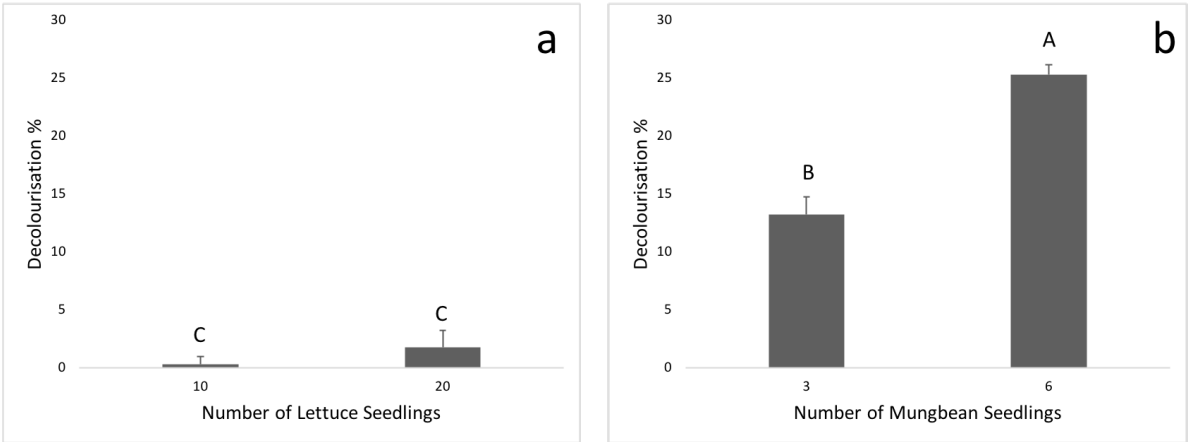

Figure 14: Effect of varying seedling number of lettuce (a) and mungbean (b) for spent water decolourisation of malachite green (1 mg/mL). *Values presented are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 3.371, p <0.001.*

As higher decolourisation was found with 6 mungbean seedlings, this number should be used for further spent water experiments (primarily for MG (1 mg/mL) solutions). Spent water from lettuce seedlings were shown to be significantly less effective for decolourisation compared to mungbean, and perhaps a longer incubation time of seeds was needed before spent water was collected.

3.3.2 Comparison of Sterile and Non-Sterile Conditions

Following the results of the previous experiment, a longer incubation period (8 days compared to 4) was used before spent water was collected. One of the treatments used in this experiment (Treatment A) had seedlings replaced with fresh mungbean seeds after 4 days, allowing two sets of 6 mungbean seedlings to be grown in the same water that would be collected after a total of 8 days. The other treatment (B) used was one set of 6 mungbean seedlings over the 8 days.

The results show a significant increase in decolourisation of malachite green (1 mg/mL) in treatment A compared to treatment B (Figure 15). This indicates that increased concentration of spent water from mungbean seeds had a higher decolourisation ability. There was no significant difference between sterile and non-sterile conditions for treatment A, and 81% decolourisation of the dye was achieved. This result suggests that there was no microbial activity assisting this decolourisation. While there was a lower decolourisation % in sterile conditions from treatment B, the decolourisation of 32% can be attributed to spent water from mungbean alone. These results show that longer incubation time, along with increased concentration of spent water, greatly enhance decolourisation.

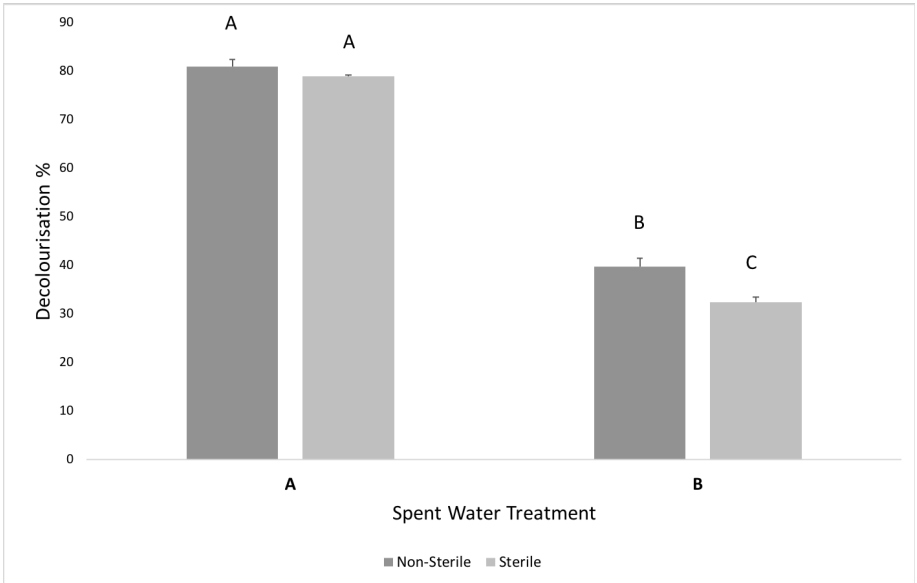

Figure 15: Decolourisation of malachite green (1 mg/mL) under sterile or non-sterile conditions. Treatment A = 6 mungbean seeds left in dH_2O for 4 days before being replaced with 6 fresh mungbean seeds and left another 4 days (8 days total), Treatment B = 6 mungbean seeds left in dH2O for 8d. *Presented values are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 3.444, p <0.001.*

Without more advanced analysis available (such as GC-MS and HPLC-MS), the specific enzymes and biomolecules present in the spent water remains unknown. However, previous studies have shown decolourisation of textile dyes by extracting various plant peroxidases for dye degradation (Kalsoom et al, 2015). One study used extracted soybean peroxidases for the treatment of toxic arylamines from dye (Mukherjee et al, 2018). It is possible that similar enzymes are active in the decolourisation of dyes in this study.

3.3.3 Effect of Concentrated Spent Water

The same approach of using concentrated spent water from treatment A in the previous experiment was repeated here for both lettuce and mungbean seedlings and both dye solutions. Decolourisation of methylene blue (0.04 mg/mL) by spent water from lettuce seedlings was 72% which was significantly higher than mungbean even though this treatment still showed a high decolourisation of 60% (Figure 16a). Spent water from lettuce was able to decolourise malachite green (1 mg/mL) by 18% while that of mungbean showed a significantly higher 77% decolourisation (Figure 16b).

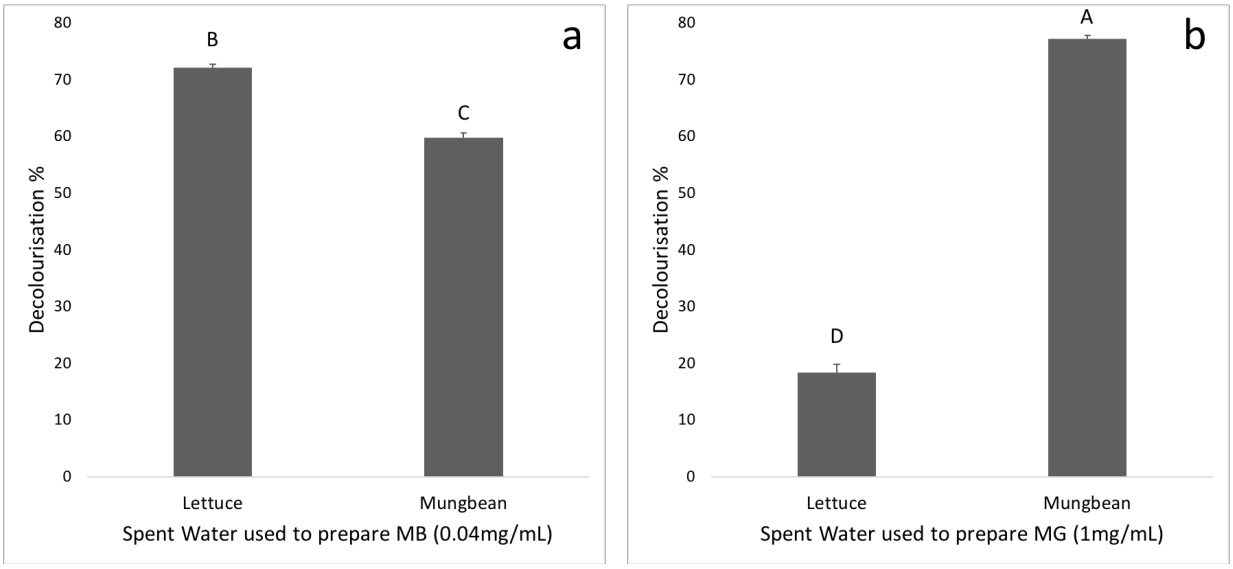

Figure 16: Decolourisation of a) methylene blue (0.04 mg/mL) and b) malachite green (1 mg/mL) prepared from spent water of lettuce (20 seeds placed in dH_2O for 4 days and replaced with 20 fresh seeds for a further 4 days (8 days total) and mungbean (6 seeds placed in dH_2O for 4 days and replaced with 6 fresh seeds for a further 4 days (8 days total). *Presented values are mean decolourisation percentages +SE (n=12). Means with different letters indicate that they are significantly different. LSD = 2.665, p <0.001.*

3.4 Oxidative Stress Experiments

There are many markers of oxidative stress that can be measured in plants. Peroxidases are one type of reactive oxygen species (ROS) detoxifying proteins, and their activity has been shown to increase in cells under abiotic stresses as a mechanism of stress tolerance to remove ROS which can build up and cause various damages including lipid peroxidation of membranes as

well as oxidative damage to proteins and DNA and RNA molecules (Mittler, 2002; Choudhury et al, 2017).

3.4.1 Peroxidase Assay

Lettuce and mungbean show a stark contrast in peroxidase activity. There was no difference in activity in lettuce seedlings exposed to either methylene blue or malachite green for 1d (Figure 17a). However, mungbean peroxidase activity was higher than lettuce even in the dH_2O control, and both MB (0.04 mg/mL) and MG (1 mg/mL) solutions were associated with significant increases in peroxidase activity compared to dH_2O and enzyme controls (Figure 17b).

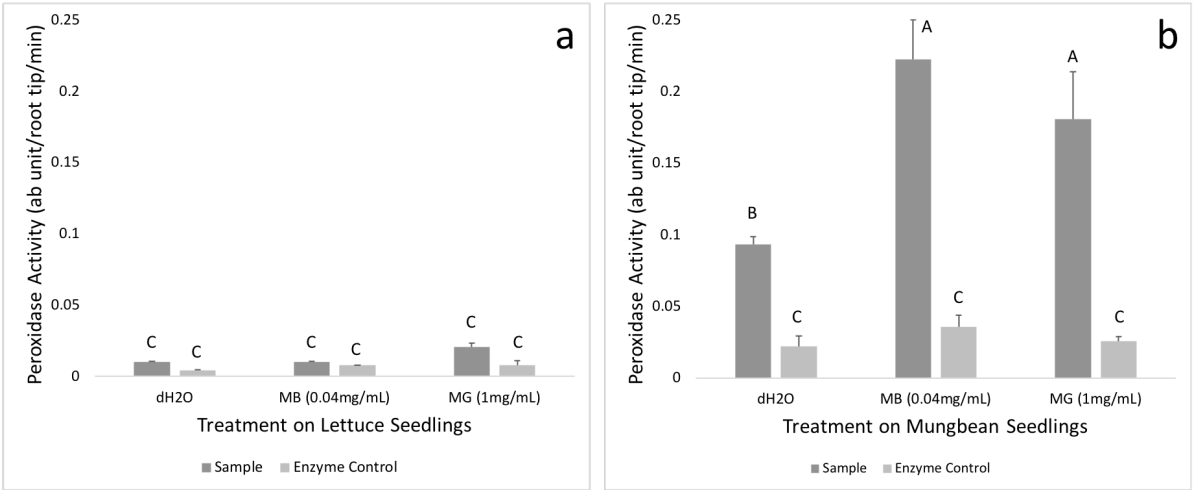

Figure 17: Peroxidase enzyme activity (ab unit/root tip/min) observed in lettuce (a) and mungbean (b) seedlings after 1 day exposure to methylene blue (0.04 mg/mL) and malachite green (1 mg/mL) compared to a dH_2O control, and individual sample enzyme controls. *Presented values are mean peroxidase activity levels (ab unit/root tip/min)+SE (n=3). Means with different letters indicate that they are significantly different. LSD = 0.045, p <0.001.*

The same trend of no increase in peroxidase activity for lettuce seedlings exposed to both dyes was found even after 3 days exposure (Figure 18a). Mungbean peroxidase activity remained significantly higher when exposed to either dye compared to dH_2O, but after 3 days the activity was significantly higher in MG (1 mg/mL) compared to MB (0.04 mg/mL) (Figure 18b).

These results show that mungbean seedlings exhibited increased peroxidase activity while under dye stress and give insight into the possible mechanism of decolourisation and potential degradation of these dyes. These findings correspond with those of many other studies which have observed increased peroxidase activity under dye stress, and during decolourisation of dyes (Patil & Jadhav, 2013; Jayanthy et al, 2014; Torbarti et al, 2014; Chandanshive et al, 2016).

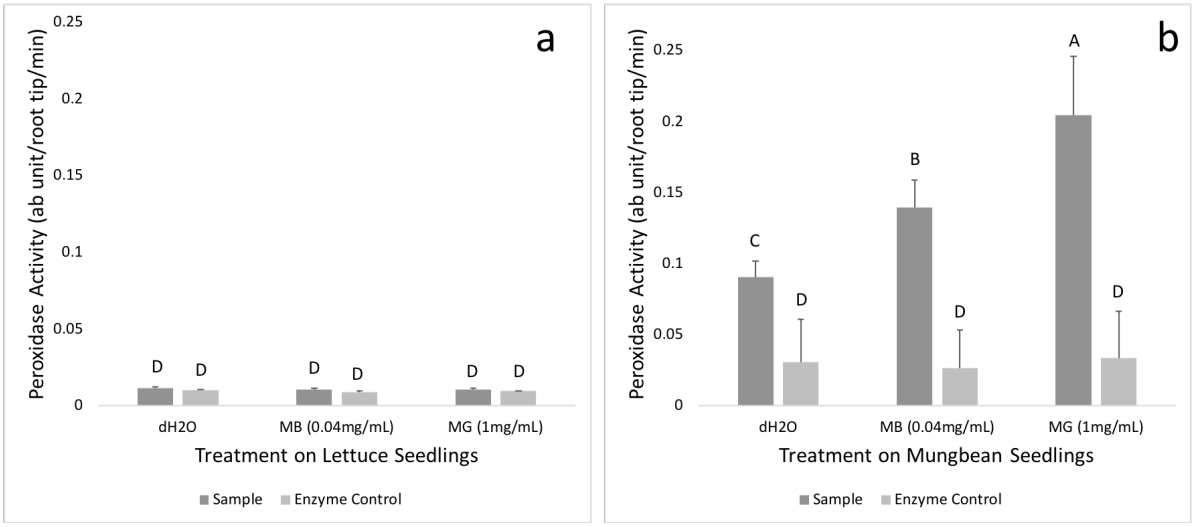

Figure 18: Peroxidase enzyme activity (ab unit/root tip/min) observed in lettuce (a) and mungbean (b) seedlings after 3 days exposure to methylene blue (0.04 mg/mL) and malachite green (1 mg/mL) compared to a dH_2O control, and individual sample enzyme controls. *Presented values are mean peroxidase activity levels (ab unit/root tip/min)+SE (n=3). Means with different letters indicate that they are significantly different. LSD = 0.038, p <0.001.*

While lettuce does not show an increase in peroxidase activity, it is possible that this plant has a higher tolerance and has not produced as many ROS requiring removal by peroxidases, or alternatively the dye is so toxic it has inhibited peroxidase formation. As lettuce has shown to have high decolourisation capability for MB (0.04 mg/mL) the first option is more likely. However, as lettuce struggles to decolourise MG at 1 mg/mL, in this case the alternative is the more probable outcome.

While stress markers such as peroxidase are a good indicator of relative stress in plants, there is a complex balancing act between the formation of ROS, and the upregulation of peroxidases and various other antioxidant enzymes and non-enzymatic antioxidants also (Waskiewicz et al, 2014). Peroxidase activity in lettuce could be indifferent due to a higher tolerance of dyes through various other antioxidant activities that were not measured in this study.

3.4.2 Lipid Peroxidation (TBARS) Assay

No difference in lipid peroxidation of lettuce was observed in either methylene blue or malachite green compared to dH_2O or the assay controls for both 1 and 3 days exposure (Figures 19a and 20a). However, there were significant increases in mungbean exposed to MB (0.04 mg/mL) and MG (1 mg/mL) compared to dH_2O and sample controls after 1 day of exposure (Figure 19b). Similar increase in lipid peroxidation have been observed by Movafeghi et al. (2016) in phytoremediation of azo dye direct blue 129 by *Spirodela polyrrhiza*, an aquatic plant.

The amount of lipid peroxidation observed in mungbean then decreases dramatically after 3 days, with both dye solutions showing significantly less lipid peroxidation than dH_2O (Figure 20b). This decrease could be explained

through mungbean developing tolerance to the dyes after 3 days, and the decolourisation and degradation of the dyes has lessened the toxicity and subsequently ROS production from the dye exposure has reduced, no longer requiring peroxidases to remove these damaging species.

Further investigation would be required to determine the exact cause of this decrease, however due to the significant increase of decolourisation after 6 days, compared to 3 days, as found in this study (**3.2.2**), it is likely that an initial stabilization within a 3 day period is required by mungbean seedlings before decolourisation can occur. This may explain the dramatic increase and subsequent drop of lipid peroxidation in dye-exposed mungbean seedlings between 1-3 days.

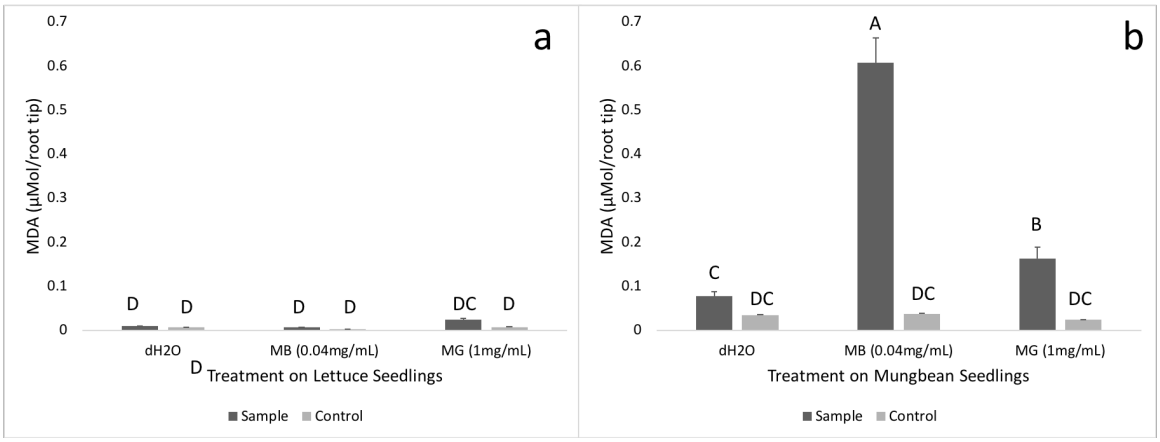

Figure 19: Lipid peroxidation level in lettuce (a) and mungbean (b) after 1 day exposure to methylene blue (0.04 mg/mL) and malachite green (1 mg/mL). Activity was measured by malondialdehyde (MDA) formation (µMol/root tip). Controls were used for each individual sample (20% TCA without TBA added to the tissue extract). *Values presented are the mean MDA formation (µMol/root tip) +SE (n=3). Means with different letters indicate that they are significantly different. LSD = 0.0538, p < 0.001.*

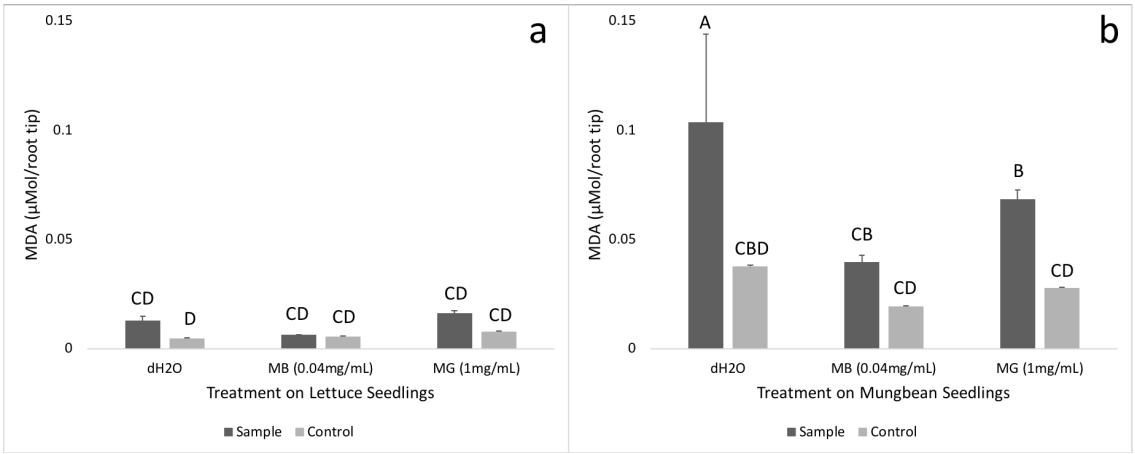

Figure 20: Lipid peroxidation level in lettuce (a) and mungbean (b) after 3 days exposure to methylene blue (0.04 mg/mL) and malachite green (1 mg/mL). Activity was measured by malondialdehyde (MDA) formation (µMol/root tip). Controls were used for each individual sample (20% TCA without TBA added to the tissue extract). *Values presented are the mean MDA formation (µMol/root tip) +SE (n=3). Means with different letters indicate that they are significantly different. LSD = 0.0345, p < 0.001.*

The lack of lipid peroxidation activity in lettuce appears to link to the similar lack of peroxidase activity, and as explained previously, could be due to the antioxidant activities of a range of other enzymes or non-enzymatic antioxidants.

3.5 Growth Measurements

There was no significant increase or decrease in radicle growth between dH_2O and methylene blue (0.04 mg/mL) or malachite green (1 mg/mL) in either lettuce or mungbean seedlings after 1 day (Figure 21a/b). While MG exposed lettuce showed less growth after 3 days (Figure 21c), this difference was not significant compared to the dH_2O control. However, there was a significant decrease in mungbean radicle growth after 3 days in both MB and MG (Figure 21d).

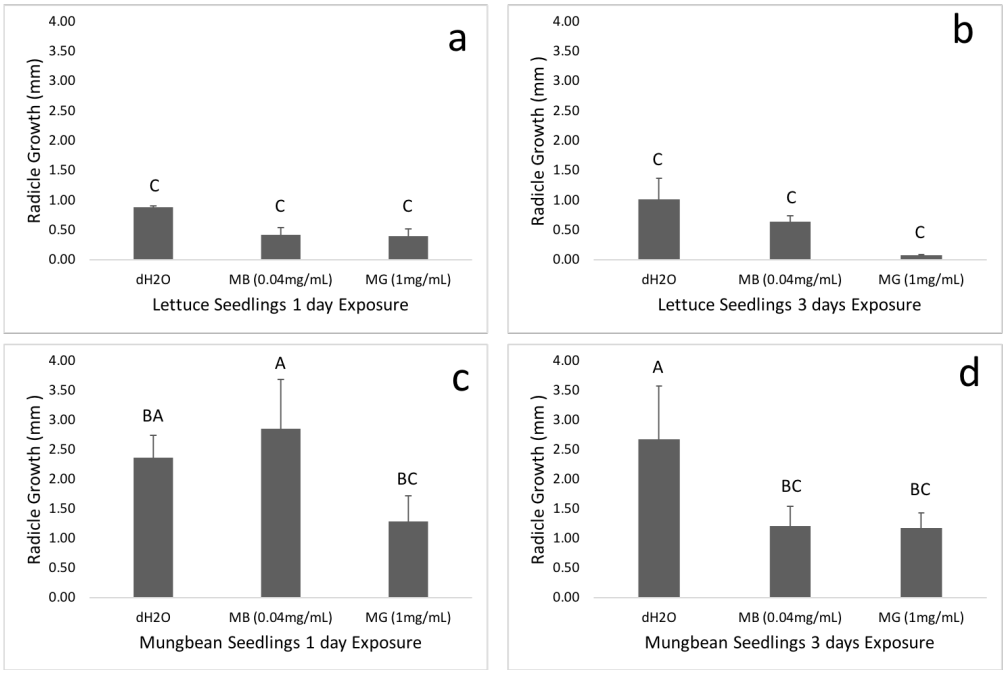

Figure 21: Effect of methylene blue (0.04 mg/mL) and malachite green (1 mg/mL) on average radicle growth (mm) in lettuce (**a** and **b**) and mungbean (**c** and **d**) seedlings after 1 day or 3 days exposure. *Presented values are the mean radicle growth (mm)+SE (n=3). Means with different letters indicate that they are significantly different. LSD = 1.242, p <0.001.*

These findings show some similarities to the oxidative stress results from peroxidase and lipid peroxidation assays, as mungbean exhibited high peroxidase activity initially, which may have led to the decrease in radicle growth after 3 days compared to dH_2O. Meanwhile, lettuce seedlings showed no significant difference in radicle growth compared to dH_2O, and similarly showed no difference in peroxidase activity or lipid peroxidation.

3.6 Phytotoxicity Evaluations

3.6.1 Direct Decolourisation

To determine if decolourised dye solutions exhibited lower toxicity than original dye solutions, the germination percentages and radicle growth of lettuce seedlings were observed between both remediated and non-remediated dyes.

There was no significant difference in germination percentage between decolourised or original methylene blue (0.04 mg/mL) solutions. However, radicle growth was found to be significantly higher in lettuce-remediated MB (0.04 mg/mL), compared to non-remediated solutions (Figure 22). This shows that the toxicity had decreased during decolourisation, reducing growth inhibition, and suggests that degradation of methylene blue might also have occurred to some extent. To confirm degradation, further analysis would be required to identify metabolites in the remediated samples, through high performance liquid chromatography (HPLC) or gas chromatography (GC) linked with mass spectroscopy (MS) methods, which were unavailable in this study due to time constraints.

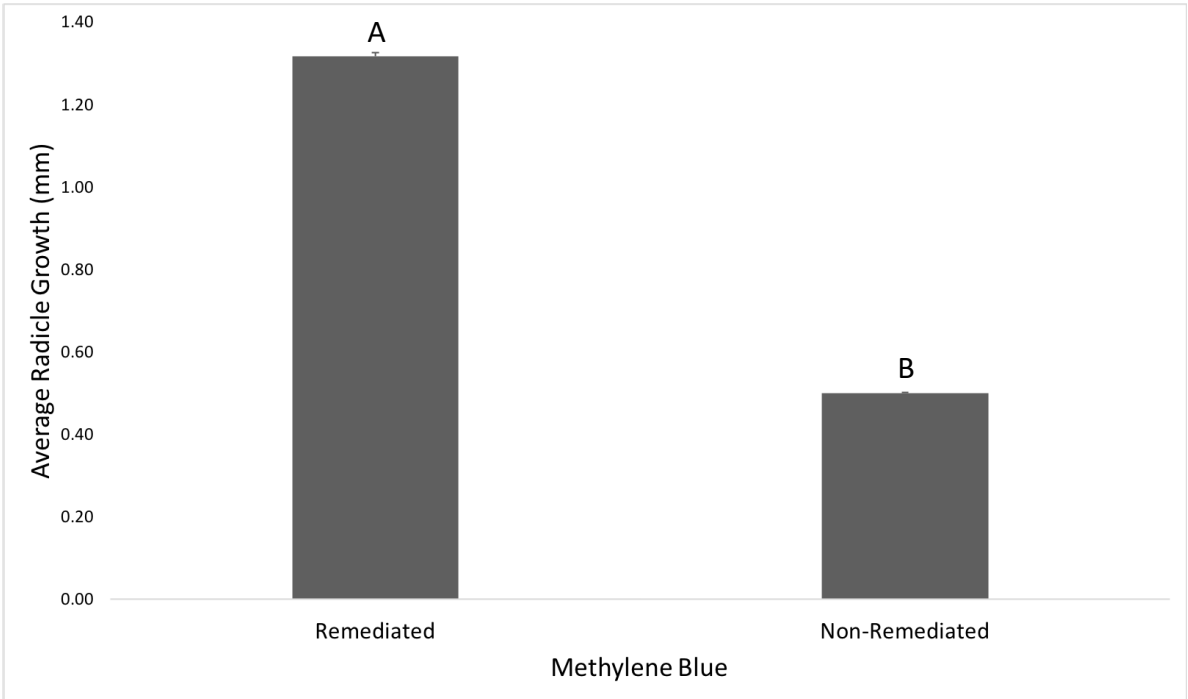

Figure 22: Radicle growth comparison between remediated and non-remediated methylene blue (0.04 mg/mL). Remediated dye was decolourised by 10 lettuce seedlings over 6 days. *Values presented are mean radicle lengths (mm) +SE (n=3). Means with different letters indicate that they are significantly different. LSD = 0.179, $p < 0.001$.*

Germination percentage was significantly higher in lettuce-decolourised malachite green (1 mg/mL) with approximately 67% germination on average compared to 47% in the original solution; a 20% increase. Both lettuce and mungbean-decolourised MG (0.1 mg/mL) solutions also showed significantly

higher germination percentages of 97% and 93% respectively when compared to the original solution (57%). These increases in germination percentage indicate lowered toxicity of these decolourised solutions, and again there was a possibility of subsequent degradation of the dyes. All significant differences in means of germination percentages listed were determined with an LSD of 14.86, CV 11.5% and 3 replicates at $p < 0.001$.

Despite mungbean showing high decolourisation of both dyes, especially malachite green, the phytotoxicity results for mungbean-decolourised solutions were inconclusive other than the increased germination percentage in the remediated 0.1 mg/mL MG. For the other dye solutions, despite significant decolourisation, mungbean seedlings did not show any difference in germination percentage or radicle growth. These results contradict what should be expected, as compared to the increased germination percentages and radicle growth from lettuce-decolourised solutions. It is likely that certain allelochemicals from mungbean have inhibited the germination and growth of lettuce seedlings in these experiments. A study by Lertmongkol et al. (2011) found *Lactuca sativa* L. germination to be severely inhibited by allelochemicals of mungbean, this has also been observed earlier by Waller et al. (1999).

Due to time constraints, alternative phytotoxicity approaches were unable to be performed for mungbean-decolourised dye solutions. However, there is a wide range of approaches used in previous studies to show decrease in toxicity of remediated dyes, such as cytogenotoxicity tests (Patil & Jadhav, 2013), as well as measuring oxidative stress markers of plant species grown in decolourised solutions compared to original dye controls (Jayanthy et al, 2014).

3.6.2 Spent Water Decolourisation

Germination percentages from lettuce seeds placed in decolourised methylene blue and malachite green (from experiment described in **2.5.4**), as well as their original solutions were compared. There was a significant increase in germination percentage of MG (1 mg/mL) decolourised by spent water from lettuce of 26.7% compared to the original solution with 16.7%. This indicates that there is a lessened toxicity related to the 20% decolourisation only, of the MG in this experiment. While mungbean spent water decolourised MG by 77%, unfortunately lettuce germination was inhibited, and phytotoxicity results were unable to be determined as the inhibition was likely from allelopathy of mungbean which was discussed previously (**3.6.1**).

There was no difference in germination percentage of MB (0.04 mg/mL) solutions, as both decolourised and original solutions showed 97-100% germination. Alternative phytotoxicity evaluation for these treatments were unable to be followed, due to time constraints.

3.7 Scanning Wavelength Measurements

3.7.1 Direct Decolourisation

Decolourisation of methylene blue and malachite green dyes by mungbean and lettuce seedlings, were confirmed with scanning wavelength spectroscopy analysis. In Figure 23, non-remediated MB (0.04 mg/mL) shows three major peaks at around 300nm (close to the UV region), and in the visible region at 600nm, and 670nm (maximum absorbance for MB). Meanwhile both lettuce and mungbean decolourised MB solutions show lower absorbance at the 300nm peak, and dramatically, while the peaks between 600-670nm have been dramatically reduced. There is a lack of new big peaks in the decolourised samples. This confirms the decolourisation of the dye by both seedlings.

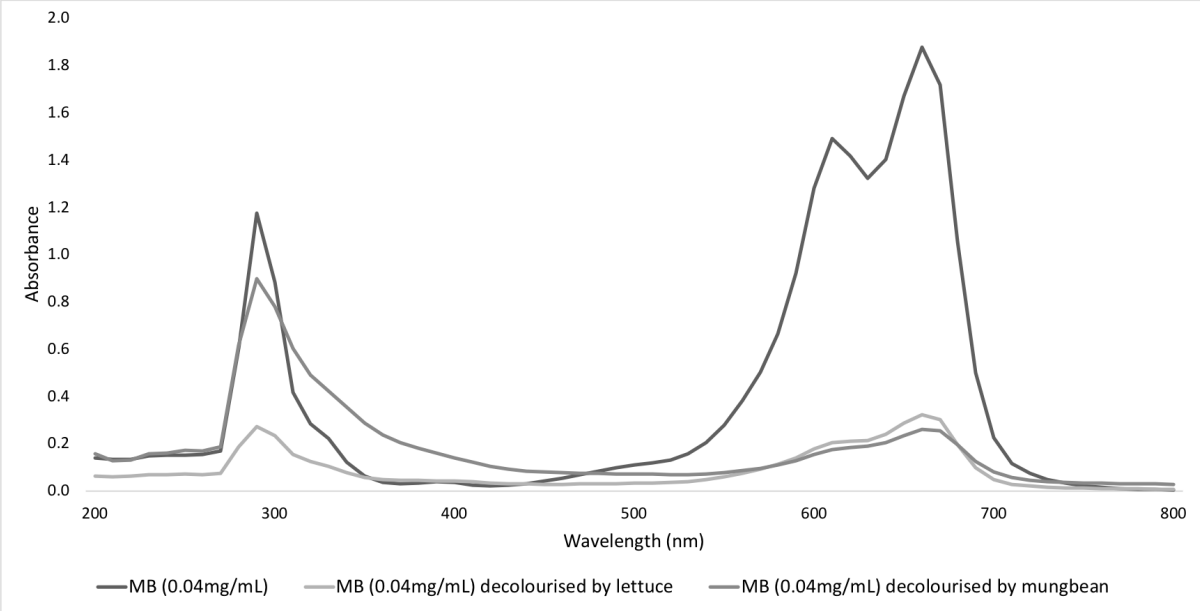

Figure 23: Spectral scan comparing methylene blue (0.04 mg/mL) with decolourised methylene blue (0.04 mg/mL) by lettuce and mungbean seedlings after 6 days. Scans were performed by a SPECTRAmax M5 Plate Reader from 200-800nm with a step of 10nm.

In Figure 24, non-remediated MG (1 mg/mL) shows three peaks, two smaller peaks around 320nm and 420nm, and a major peak at 619nm (the maximum absorbance for malachite green). MG decolourised by lettuce only shows a minor decrease in absorbance for each of these peaks, however, all peaks have been removed in MG decolourised by mungbean, confirming the decolourisation.

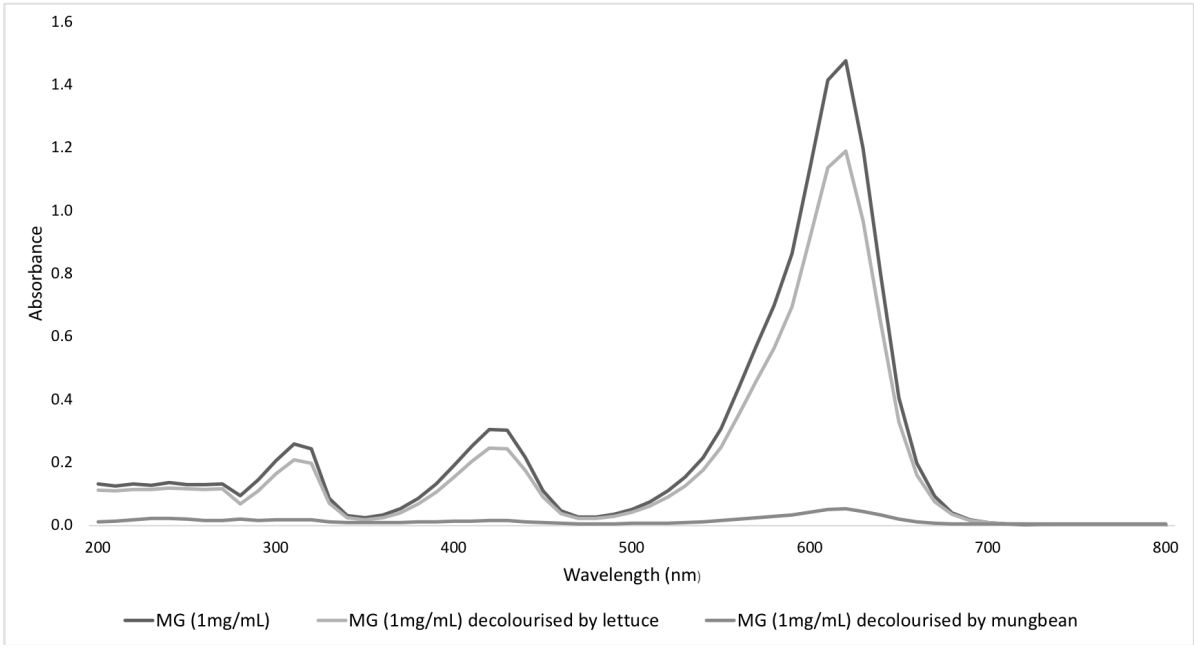

Figure 24: Spectral scans comparing malachite green (1 mg/mL) with decolourised malachite green (1 mg/mL) by lettuce and mungbean seedlings after 6 days. Scans were performed by a SPECTRAmax M5 Plate Reader from 200-800nm with a step of 10nm.

In Figure 25, non-remediated MG (0.1 mg/mL) solution is shown to have 3 peaks, in the same locations as MG (1 mg/mL). With this lowered concentration, lettuce decolourised MG has been able to decrease dramatically, showing high decolourisation, and mungbean decolourised MG shows removal of each peak, confirming the decolourisation.

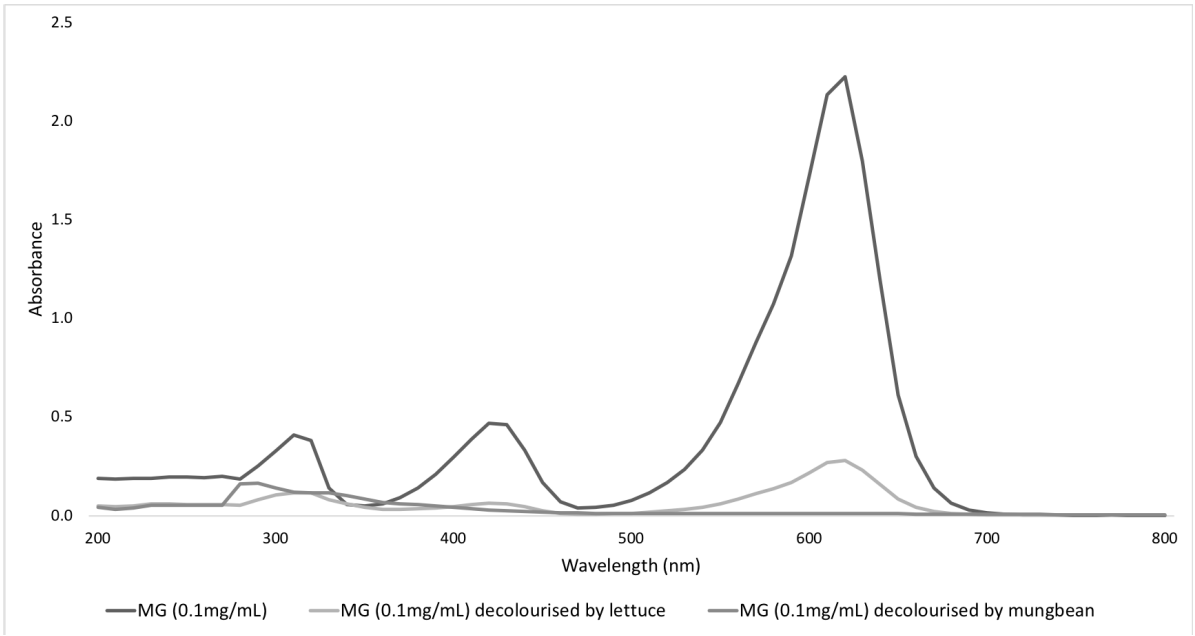

Figure 25: Spectral scans comparing malachite green (0.1 mg/mL) with decolourised malachite green (0.1 mg/mL) by lettuce and mungbean seedlings after 6 days. Scans were performed by a SPECTRAmax M5 Plate Reader from 200-800nm with a step of 10nm.

3.7.2 Spent Water Decolourisation

In Figure 26, methylene blue (0.04 mg/mL) solution before decolourisation exhibits three major peaks, in the same locations as the spectral scan for MB previously (around 300nm, 600nm, and 670nm). While both lettuce and mungbean decolourised samples show a dramatic decrease at the 600-670nm peaks, confirming decolourisation, there is a major peak from 300-400nm in the mungbean solution. There were clearly certain substances present in this spent water solution that are visible within this region. While it is unknown what these substances were without further analysis, it is possible that they were related to the decolourisation ability of concentrated mungbean spent water for this particular dye.

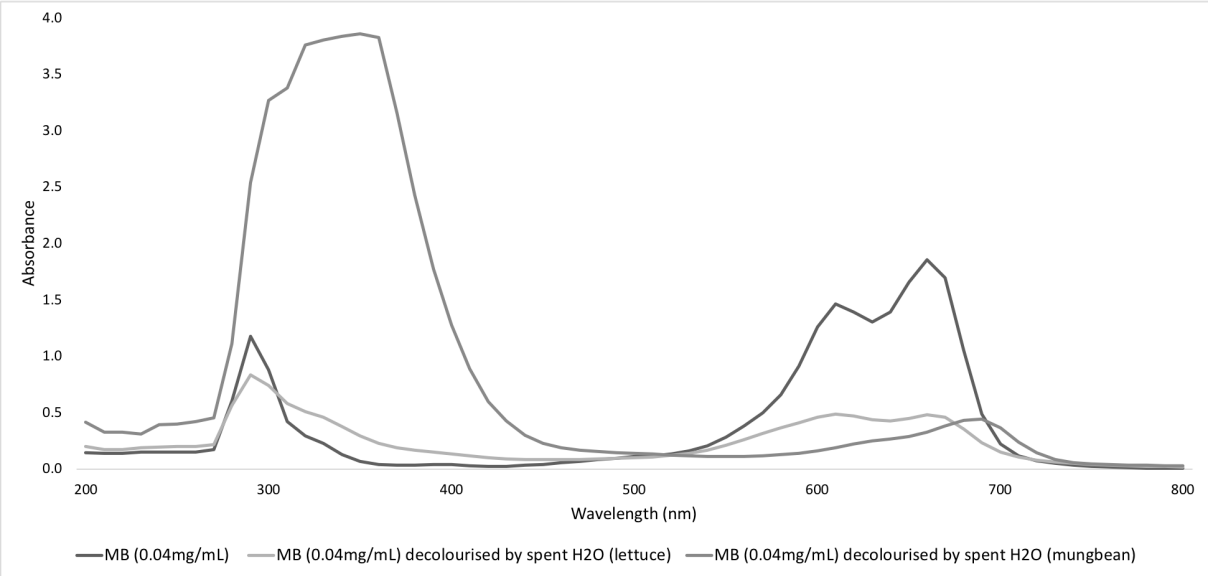

Figure 26: Spectral scans comparing methylene blue (0.04 mg/mL) with spent water decolourised methylene blue (0.04 mg/mL) by lettuce and mungbean seedlings after a total of 8 days. Scans were performed by a SPECTRAmax M5 Plate Reader from 200-800nm with a step of 10nm.

In Figure 27, non-remediated malachite green (1 mg/mL) shows three peaks at the same locations as in the previous spectral scans for MG (around 320, 420, and 619nm). There is only a slight decrease in lettuce decolourised MG, meanwhile the peaks are removed in the mungbean decolourised MG solution, confirming decolourisation.

Similar results for MB (0.04 mg/mL) decolourisation were observed by Dhaneshwar (2016). In all spectral scans where peaks have been removed in decolourised samples, especially at the maximum absorbance regions (670nm for MB and 619nm for MG), it is likely that degradation of the dyes by lettuce and/or mungbean seedlings has occurred. However, this cannot be confirmed without further analysis such as GC-MS and HPLC-MS, or Fourier Transform Infrared Spectroscopy (FTIR) which have been used in many studied on dye removal (Torbarti et al, 2014; Chandanshive et al, 2016; Gupta et al, 2016), to confirm degradation along with decolourisation.

Further confirmation is especially important as sometimes metabolism of synthetic dyes can result in the formation of other toxic compounds (Gürses et al, 2016), which are not the desired result from decolourisation and detoxification of dyes.

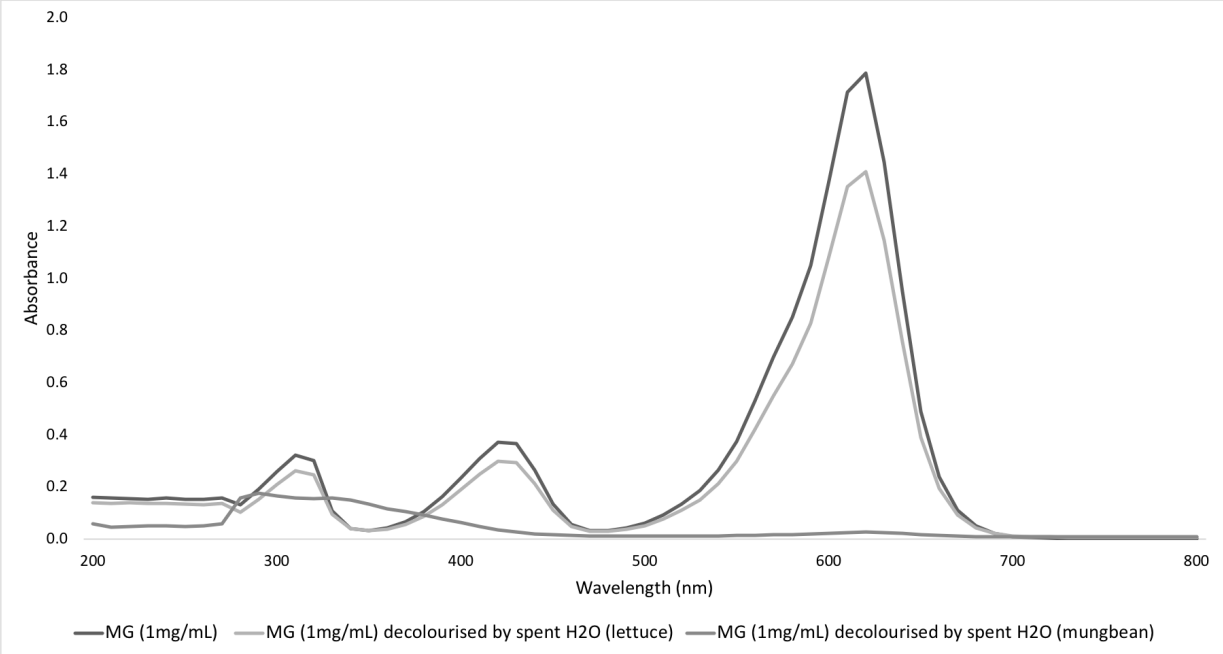

Figure 27: Spectral scans comparing malachite green (1 mg/mL) with spent water decolourised malachite green (1 mg/mL) by lettuce and mungbean seedlings after a total of 8 days. Scans were performed by a SPECTRAmax M5 Plate Reader from 200-800nm with a step of 10nm.

4. Final Conclusions and Future Directions

4.1 Key Findings

In this research, the aim was to determine if fast germinating plants, including mungbean (*Vigna radiata*) and lettuce (*Lactuca sativa*, L), would be useful in phytoremediation of environmental pollutants such as toxic synthetic dyes; malachite green and methylene blue. The findings from this study allow the conclusion that both lettuce and mungbean seedlings can effectively decolourise malachite green at 0.1 mg/mL and methylene blue at 0.04 mg/mL over six days incubation at room temperature in either light or dark conditions. Mungbean has also been shown to be highly effective in the decolourisation of malachite green at 1 mg/mL under the same conditions. Lettuce in particular showed enhanced decolourisation for methylene blue decolourisation, while mungbean demonstrated this for malachite green – showing that these plants could each be targeted towards specific dyes. Spent water from mungbean, in concentrated doses, was also shown to effectively decolourise both malachite green at 1 mg/mL and methylene blue at 0.04 mg/mL over 4 days incubation at room temperature. Spent water from lettuce, in concentrated doses, was found to effectively decolourise methylene blue at 0.04 mg/mL under the same conditions.

This study also found a decreased toxicity of remediated dyes through phytotoxicity evaluations of germination percentage and radicle growth. Increased peroxidase activity was observed in mungbean seedlings exposed to both dyes over after 1-3 days. Lipid peroxidation in mungbean was also found to increase after 1 day but subsequently decrease after 3 days. These findings indicate activation of antioxidant enzymes under dye stress. Lettuce seedlings appeared more tolerant than mungbean under dye stress. Spectral scans of decolourised dyes compared to original dye solutions, confirmed the dye removal.

These findings show that both mungbean and lettuce seedlings have great promise as fast-germinating plants to be used for phytoremediation of environmental pollutants, especially for the two toxic synthetic dyes used in this study: malachite green and methylene blue. Phytoremediation has many significant benefits over alternative dye removal methods such as bioremediation, and physio-chemical approaches. These include: the low cost (particularly with common plants such as lettuce and mungbean), being an attractive eco-friendly "green" approach, and no addition of chemicals. Phytoremediation also has another advantage over bioremediation, as many textile dyes exhibit antimicrobial activity.

In conclusion, the findings of this study reveal the potential of lettuce and mungbean seedlings for effective decolourisation of methylene blue and malachite green. Adsorption of dye to seedings and biodegradation are possible mechanisms responsible for decolourisation of dye solutions in this study.

4.2 Limitations of this Study

Only a marker of oxidative stress (lipid peroxidation) and activity of a general antioxidative enzyme (guaiacol peroxidase) were investigated here. Dye-triggered oxidative stress causing germination inhibition and decreased growth of seedlings as shown in this study, could be the result of multiple reactive oxygen species (ROS) and the activities of several antioxidative enzymes. For example, laccase and catalase have been shown to increase under dye stress of azo dyes (Chandanshive et al, 2016; Torbarti et al, 2014) along with other enzymes and also non-enzymatic antioxidants including ascorbic acid, more commonly known as Vitamin C (Waskiewicz et al, 2014). We could have also looked into proline content, an amino acid which commonly accumulates in plants exposed to abiotic stresses (Kaur & Asthir, 2015), for example, osmotic stress, of lettuce and mungbean.

Results for phytotoxicity evaluations of methylene blue and malachite green decolourised by mungbean were inconclusive in this study. This was most likely due to the allelopathic nature of mungbean seedlings, which inhibited germination and growth of lettuce seeds used in these experiments. An alternative approach for future phytotoxicity evaluations would be needed.

Investigation into the mechanism of dye decolourisation by lettuce and mungbean seedlings, and quantifying the degradation of these toxic dyes through methods such as gas chromatography mass spectrometry (GC-MS) analysis or high performance liquid chromatography (HPLC) to identify metabolites in decolourised samples, would further determine the effectiveness of these plants for phytoremediation of methylene blue and malachite green. However, this study was time-limited due to only having 12 months available for topic investigation, literature review, writing the research proposal, conducting preliminary experiments and the main experiments, data analysis and writing up the thesis.

4.3 Future Research Suggestions

Lettuce and mungbean seedlings have been shown in this research to exhibit a high capacity for decolourisation of methylene blue and malachite green under certain conditions, especially lettuce for the targeted decolourisation of methylene blue, and mungbean for malachite green. More research could be conducted using these two plant species to investigate their effectiveness on the decolourisation of other toxic synthetic dyes and/or direct effluent from dye-polluted sites.

Further investigation is needed to confirm the degradation of toxic metabolites present in both dyes, after decolourisation. While this study found several indications of decreased dye toxicity after decolourisation by lettuce and mungbean seedlings, more advanced analysis of dye solutions (such as GC-MS or HPLC) before and after decolourisation are needed to

ensure that this promising phytoremediation approach is effective for the detoxification of methylene blue and malachite green, as well as their decolourisation.

Research could also be conducted to determine the metabolic pathway for likely decolourisation and degradation of methylene blue and malachite green dyes by lettuce and mungbean. Knowledge of the specific enzymes involved in the process would enhance understanding of how to optimize conditions for decolourisation even further. Extraction of targeted enzymes from these seedlings could also prove to be a useful tool for textile effluent decontamination.

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